

# **Isolierung und Charakterisierung von Plasmazellen des Haushuhns (*Gallus gallus*)**

von

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Inaugural-Dissertation zur Erlangung der Doktorwürde der

Tierärztlichen Fakultät der Ludwig-Maximilians-Universität

München

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aus

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*Meinem Vater, der die Fertigstellung dieser Arbeit  
nicht mehr miterleben konnte*

*Danke für Alles!*

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## Abkürzungsverzeichnis

|               |                                                                 |
|---------------|-----------------------------------------------------------------|
| AID           | aktivierungsinduzierte Cytidin-Desaminase                       |
| APRIL         | A proliferation-inducing ligand                                 |
| BAFF          | B cell activating factor belonging to the TNF family            |
| BAFF-R        | B cell activating factor belonging to the TNF family – Rezeptor |
| BCL6          | B-cell lymphoma 6                                               |
| BCMA          | B cell maturation antigen                                       |
| BCR           | B cell receptor                                                 |
| BF            | Bursa fabricius                                                 |
| BLIMP-1       | B lymphocyte-induced maturation protein-1                       |
| BrdU          | Bromdesoxyuridin                                                |
| BSAP          | B-cell specific activator protein                               |
| CAML          | Calcium modulating cyclophilin ligand                           |
| CCR7          | C-C chemokine receptor type 7                                   |
| CD21          | Cluster of Differentiation 21                                   |
| CD40          | Cluster of Differentiation 40                                   |
| CD40L         | Cluster of Differentiation 40 – Ligand                          |
| CSR           | Klassenwechsel-Rekombination                                    |
| CXCL12        | CXC-Motiv-Chemokin 12                                           |
| CXCL13        | CXC-Motiv-Chemokin 13                                           |
| CXCR4         | C-X-C chemokine receptor type 4                                 |
| CXCR5         | C-X-C chemokine receptor type 5                                 |
| EALT          | eye associated lymphatic tissue                                 |
| EBF-1         | Early B-cell factor-1                                           |
| ER            | endoplasmatisches Retikulum                                     |
| FACS          | Fluorescence Activated Cell Sorting                             |
| HG            | Harderian Gland                                                 |
| IBDV          | Infectious Bursal Disease Virus                                 |
| IBV           | Infektiöses Bronchitis Virus                                    |
| IFN- $\alpha$ | Interferon alpha                                                |

|               |                                             |
|---------------|---------------------------------------------|
| IFN- $\gamma$ | Interferon-gamma                            |
| Ig            | Immunglobulin                               |
| IgA           | Immunglobulin A                             |
| IgD           | Immunglobulin D                             |
| IgE           | Immunglobulin E                             |
| IgG           | Immunglobulin G                             |
| IgM           | Immunglobulin M                             |
| IgY           | Immunglobulin Y                             |
| IL-10         | Interleukin 10                              |
| IL-2          | Interleukin 2                               |
| IL-21         | Interleukin 21                              |
| IL-6          | Interleukin 6                               |
| LLPC          | Long lived plasma cell                      |
| MACS          | Magnetic Activated Cell Sorting             |
| MALT          | mucosa associated lymphatic tissue          |
| MHCII         | Major histocompatibility complex class II   |
| NALT          | nasal associated lymphatic tissue           |
| NDV           | Newcastle-Disease-Virus                     |
| NK-Zellen     | natürliche Killerzellen                     |
| Pax-5         | Paired box protein 5                        |
| slg           | surface immunoglobulin                      |
| SLPC          | Short lived plasma cell                     |
| TACI          | transmembrane activator and CAML-interactor |
| Tfh           | T follicular helper cells                   |
| TGF- $\beta$  | Transforming Growth Factor-beta             |
| TNF           | Tumor necrose factor                        |
| Tregs         | Regulatorische T-Zellen                     |

# 1 Einleitung

Geflügel, insbesondere Hühner, spielt in Deutschland eine bedeutende wirtschaftliche Rolle. Im Jahr 2023 wurden 156 Millionen Hühner gehalten (1), die hauptsächlich zur Lebensmittelproduktion dienen, wobei die Eierproduktion, allein auf Betriebe mit mehr als 3000 Haltungsplätzen bezogen, bei 13,1 Milliarden Stück lag (2).

Da der Einsatz von Medikamenten bei lebensmittelliefernden Tieren streng reglementiert ist, kommt der Infektionsprävention eine besondere Bedeutung zu. Eine effektive Kontrolle von Krankheitserregern wird daher in der Wirtschaftsgeflügelhaltung maßgeblich durch den Einsatz von Vakzinen gewährleistet.

Neben ihrer wirtschaftlichen Bedeutung haben sich Hühner auch als wichtige Modelltierart für die immunologische Forschung etabliert. Viele grundlegende Erkenntnisse über das Immunsystem, wie die Unterteilung in B („*bursa-derived*“)- und T („*thymus-derived*“)-Lymphozyten sowie die Diversifizierung der B-Zell-Rezeptoren, stammen aus der Forschung an Hühnern (3). Das aviäre Immunsystem weist dabei einige wesentliche Unterschiede zu dem der Säuger auf, insbesondere in Bezug auf die B-Zell-Entwicklung (4, 5).

Die Bursa Fabricii, das einzigartige primäre B-Zellorgan der Vögel, spielt eine zentrale Rolle bei der Reifung und Diversifizierung der B-Zellen. Diese sind für die humorale Immunantwort und die Produktion von Antikörpern essenziell, die einen entscheidenden Beitrag zum Schutz gegen bakterielle und virale Erreger leisten (6).

Ein besseres Verständnis der Mechanismen, die die B-Zell-Differenzierung und Antikörperproduktion regulieren, ist essenziell, um aktuelle Impfstoffe und Impfstrategien gegen die aviäre Influenza weiterzuentwickeln (7). Dies ist entscheidend, um die Gesundheit großer Geflügelbestände langfristig zu gewährleisten.

Trotz bedeutender Fortschritte in der Hühnerforschung gibt es weiterhin offene Fragen zu den molekularen Mechanismen, die die Entwicklung und Funktion von B-Zellen nach dem Verlassen der Bursa Fabricii steuern. Besonders für die terminalen Differenzierungsstufen der B-Zellen, namentlich Memory- und Plasmazellen, fehlen spezifische Oberflächenmarker. Diese Lücke erschwert nicht nur die genaue Charakterisierung dieser Zelltypen erheblich, sondern verhindert auch die Untersuchung der zugrunde liegenden molekularen Prozesse.



Die fortlaufende Forschung in diesem Bereich ist entscheidend, um nicht nur das Verständnis des aviären Immunsystems zu vertiefen, sondern auch die Anwendung der Erkenntnisse zur Verbesserung der Geflügelgesundheit zu ermöglichen.

Daher untersucht diese Arbeit Möglichkeiten Plasmazellen *in-vitro* zu generieren und ein markerbasiertes Isolationsprotokoll für Plasmazellen zu entwickeln, um damit die Grundlage für eine tiefergehende Charakterisierung dieser Zellen zu schaffen.

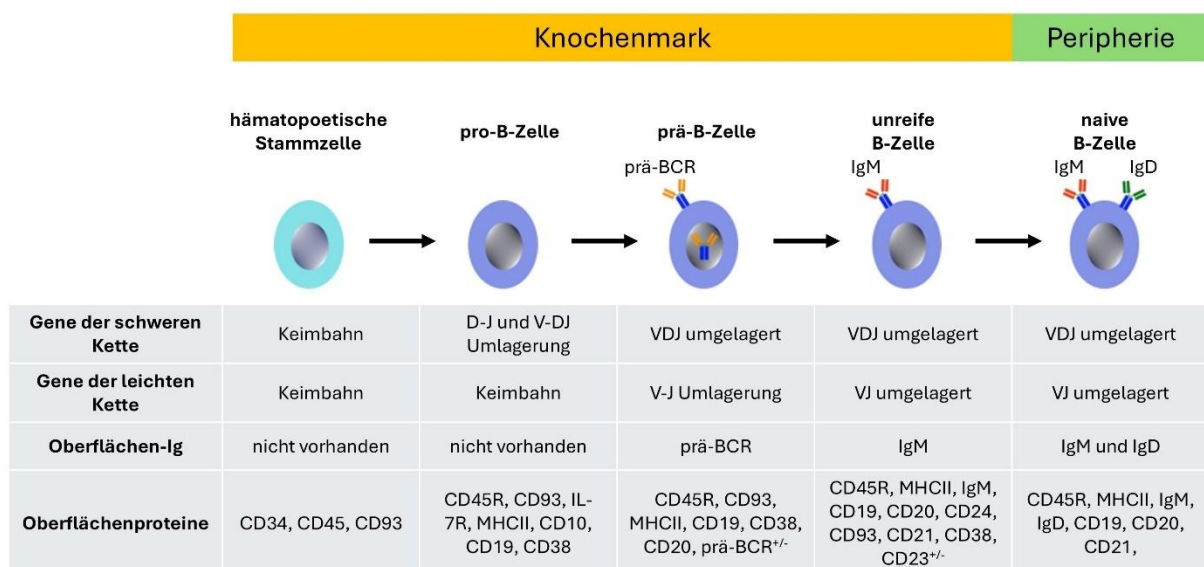
## 2 Literaturübersicht

B-Zellen sind eine wesentliche Komponente des adaptiven Immunsystems, die für die Produktion von Antikörpern verantwortlich sind. Diese Antikörper erkennen und binden spezifische Antigene auf der Oberfläche von Krankheitserregern wie Viren, Bakterien oder deren Bestandteile und Produkte. Bei der Bindung eines Antikörpers an ein Antigen wird das Pathogen markiert und kann im Anschluss durch verschiedene Mechanismen des Immunsystems zerstört oder neutralisiert werden. B-Zellen spielen außerdem eine Rolle bei der Bildung des immunologischen Gedächtnisses, welches den Körper langfristig vor erneuten Infektionen schützt.

### 2.1 Entwicklung des B-Zell-Systems beim Säuger

Die B-Zell-Entwicklung beim Säuger verläuft in zwei Phasen: die Reifung von der hämatopoetischen Stammzelle zur unreifen B-Zelle im Knochenmark (**Abbildung 1**) sowie die finale Differenzierung in den peripheren lymphatischen Organen wie beispielsweise der Milz (**Abbildung 2**) (8, 9).

#### 2.1.1 Von der hämatopoetischen Stammzelle zur unreifen B Zelle – Entwicklung im Knochenmark



**Abbildung 1** Überblick der B-Zell-Entwicklung (8-15)

Aus einer hämatopoetischen Vorläuferzelle entwickelt sich bei Menschen und Mäusen im Knochenmark eine gemeinsame lymphatische Vorläuferzelle aus welcher sich B- und T-Lymphozyten, sowie die natürliche Killerzellen (NK-Zellen) entwickeln (16, 17).

Durch Wechselwirkungen mit Stromazellen und spezifischen Wachstumsfaktoren (z. B. E2A, EBF) wird die B-Zell-Entwicklung eingeleitet (18, 19). Die dabei stattfindende Rekombination der Immunglobulin-Gene ermöglicht die Ausbildung der Vielfalt des B-Zell-Rezeptors (BCR), wobei fehlerhafte Rekombinationen eliminiert werden (10, 20, 21). Nach erfolgreicher Umlagerung der leichten und schweren Ketten des BCRs folgt eine Selektion: nur funktionsfähige und nicht autoreaktive B-Zellen überleben (15, 22).

### **2.1.2 Ausreifung der B-Zellen in der Peripherie**

Nach der Reifung im Knochenmark verlassen die B-Zellen das Organ und migrieren in periphere lymphatische Organe wie beispielsweise die Milz (23). Hier passieren sie Übergangsstadien, die durch die Expression von CD21, einem Komplementrezeptor und Bestandteil des B-Zell-Corezeptors (24), unterschieden werden (T1 = CD21 negativ; T2 = CD21 positiv) (25).

Damit die Zellen die Übergangsstadien durchlaufen und schließlich Teil des peripheren B-Zell-Pools werden können, sind schwache Signale über den BCR notwendig. Diese Signale sind wesentlich schwächer als jene, die bei einer Bindung des Oberflächen-IgM des BCR an ein körperfremdes Antigen entstehen und werden möglicherweise über niedrigaffine Erkennung von körpereigenem Antigen ausgelöst. Der genaue Mechanismus ist jedoch bislang unbekannt (25-28).

Während einige B-Zellen als Marginalzonen-B-Zellen in der Milz verbleiben, zirkuliert der Großteil als follikuläre B-Zellen und siedelt sich später wieder in B-Zell-Arealen der Milz oder anderer peripherer lymphatischer Organe, wie den Lymphknoten, an (23, 29).

Dort erhalten sie, vermittelt über das Zytokin BAFF (B cell activating factor belonging to the TNF family), das mit den Oberflächenrezeptoren BAFF-R (BAFF Rezeptor) (30), BCMA (B cell maturation antigen) (31) oder TACI (transmembrane activator and CAML-interactor) (32) interagieren kann, Überlebenssignale (33-35).

### 2.1.3 B-Zell-Subtypen

Beim Säuger kann zwischen B1, follikulären (B2) und Marginalzonen-B-Zellen unterschieden werden.

Die B1-B-Zellen befinden sich im Gegensatz zu den B2- und Marginalzonen-B-Zellen Bestandteil der angeborenen und nicht der adaptiven Immunantwort. Sie finden sich hauptsächlich in der Pleura- und der Peritonealhöhle und weniger in den peripheren lymphatischen Organen. Zusätzlich unterscheidet diese, dass sie im Gegensatz zu den anderen B-Zellen, kein, beziehungsweise nur wenig CD45 exprimieren. Sie entstehen hauptsächlich vor der Geburt in der fetalen Leber und sind in der Lage ohne T-Zell-Hilfe Antikörper vom IgM-Isotyp zu produzieren (36-38).

B2- oder follikuläre B-Zellen befinden sich in der Milz und anderen lymphatischen Organen, die Marginalzonen-B-Zellen befinden sich hauptsächlich in der namensgebenden Grenzzone zwischen weißer und roter Milzpulpa. Im Gegensatz zu den B1-Zellen stammen beide B-Zell-Subtypen aus dem Knochenmark und ihr Überleben ist von BAFF abhängig. Marginalzonen-B-Zellen können im Gegensatz zu den follikulären B-Zellen auch ohne T-Zell-Hilfe Antikörper produzieren und so, ähnlich wie die B1-Zellen schnell reagieren, produzieren aber auch hauptsächlich Antikörper vom IgM-Isotyp (25, 27, 39-41).

### 2.1.4 Differenzierung von Antikörper-produzierenden Plasmazellen

Nach Antigenkontakt exprimieren naive B-Zellen den Chemokinrezeptor CCR7 (C-C chemokine receptor type 7). Dies steuert ihre Migration zur Grenze zwischen den B- und T-Zell-Zonen der lymphatischen Strukturen (42, 43).

An der Grenze zwischen B- und T-Zell-Zone kommt es zur Interaktion zwischen aktivierter B- und T-Zelle. Die Interaktion über das Rezeptor-Liganden-Paar CD40 – CD40L steigert das Überleben von B-Zellen und induziert die Expression weiterer costimulatorischer Moleküle, sowie die Zellproliferation (44).

Nach zwei bis drei Tagen nimmt die Expression von CCR7 ab und die B-Zellen wandern von der Grenze zur T-Zell Zone in die interfollikulären Zonen zurück (45). Ein Teil der B-Zellen lagern sich dort zu Aggregaten von proliferierenden Zellen, sogenannten

primären Foci, in denen sie sich zu Antikörper-produzierenden Plasmablasten differenzieren, zusammen.

Der andere Teil der aktivierten B-Zellen wandert zusammen mit seinen assoziierten T-Zellen in die primären lymphoiden Follikel. Dort proliferieren sie weiter und bilden letztlich Keimzentren (GC, germinal center) (46, 47), die sich in eine dunkle Zone mit mitotisch aktiven Zentroblasten und eine helle Zone aus Zellen mit geringer mitotischer Aktivität unterteilen lassen. In ihnen kommt es zur Affinitätsreifung und zum, durch die aktivierungsinduzierte Cytidin-Desaminase (AID) induzierten, Immunglobulin-Klassenwechsel (48, 49). Während dieser Prozesse wandern die Zellen zwischen dunkler Zone (Ort der Proliferation) und heller Zone (Ort der Selektion) hin und her. Diese räumliche Organisation wird durch Chemokinrezeptoren wie CXCR4 (C-X-C chemokine receptor type 4) und CXCR5 (C-X-C chemokine receptor type 5) in Interaktion mit ihren Liganden, CXCL12 (CXC-Motiv-Chemokin 12) und CXCL13 (CXC-Motiv-Chemokin 13) reguliert (50, 51). Die B-Zellen verlassen die GCs in der Regel als bereits antikörpersezernierende, aber noch teilungsfähige Plasmablasten und wandern dann in verschiedene Gewebe, wie zum Beispiel das Knochenmark aus, wo sie sich zu reifen, nicht mehr teilungsfähigen Plasmazellen ausdifferenzieren (52).

Auf Grund der Lokalisation der Bildung von Plasmablasten/Plasmazellen kann also zwischen einer extrafollikulären und follikulären Antikörperantwort unterschieden werden.

### **2.1.5 Mikroskopischer Phänotyp von Plasmazellen**

Plasmazellen weisen eine charakteristische Morphologie auf und konnten anhand dieser, bereits vor dem Einsatz von spezifischen Antikörpern, mikroskopisch identifiziert werden. Im Knochenmark des Menschen zeigen sich Plasmazellen als rundlich bis ovale Zellen mit einer Größe von 10-30µm und basophilem Zytoplasma. Der Zellkern ist exzentrisch gelegen und lässt, verursacht durch dicht zusammengelagertes Chromatin, eine charakteristische Wagenradstruktur erkennen. Mit May-Grünwald-Giemsa-Färbung angefärbte PCs zeigen um den Zellkern einen farblosen Hof, der dem ungefärbten Golgi-Apparat entspricht.

In elektronenmikroskopischen Analysen zeigt sich eine feine villinöse Zellmembran. Gelegentlich lässt sich ein mittlerer Nukleolus nachweisen. Das raue endoplasmatische Retikulum (rER) ist stark ausgeprägt und konzentrisch um den Zellkern angeordnet, wobei zahlreiche Ribosomen an der Außenseite haften. Diese Struktur weist auf die hohe Synthesekapazität für Immunglobuline hin. In unmittelbarer Kernnähe befindet sich ein gut entwickelter Golgi-Apparat. Zudem lassen sich gelegentlich kleine zytoplasmatische Einschlüsse nachweisen (53).

### **2.1.6 Kurzlebige Plasmazellen**

Neben ihrer Lokalisation und dem produzierten Antikörper-Isotyp, können Plasmazellen auch anhand ihrer Lebensspanne unterteilt werden. Die sogenannten kurzlebigen Plasmazellen (SLPCs, short lived plasma cells) entstehen mit Hilfe von follikulären T-Helfer-Zellen (Tfh) in den extrafollikulären Immunantworten innerhalb von ca. vier bis sieben Tagen nach Antigenkontakt (54-56). Sie können nach Infektionen oder Impfungen schnell große Mengen von niedrig-affinen IgM- und teils IgG-Antikörpern produzieren (57, 58), sterben jedoch nach wenigen Tagen wieder ab (59, 60). Die relativ kurze Überlebensspanne dieser Zellen, kann zum Teil darauf zurückgeführt werden, dass es im Rahmen der intensiven Antikörperproduktion zu Stressreaktionen im endoplasmatischen Retikulum (ER) kommt, welche die Apoptose der Zellen induzieren. Zwar wird die Aktivierung von für die Apoptose wichtigen Enzymen wie die Caspasen 3 und 9 im Rahmen der Ausdifferenzierung zu SLPCs blockiert, jedoch kann dies das Absterben der Zellen nicht verhindern (59).

Die SLPCs sind in der roten Milzpulpa, sowie den Marksträngen der Lymphknoten lokalisiert (61-63) und spielen vor allem in der Frühphase einer Infektion eine wichtige Rolle bei deren Eindämmung. Bei einigen Autoimmunerkrankungen, wie der rheumatoiden Arthritis oder Lupus erythematosus dominieren sie die frühe Autoantikörperantwort (64, 65).

### **2.1.7 Langlebige Plasmazellen**

Parallel zur initialen B-Zell-Antwort durch die SLPCs entstehen in der GC-Reaktion mit Hilfe anderer Zellen sowie weiterer Faktoren (66-68) Plasmazellen, die hochaffine Antikörper produzieren (64, 69).

Ein Teil dieser Plasmazellen wird zu langlebigen Plasmazellen (LLPCs, long lived plasma cells) (70), die in der Lage sind, kontinuierlich Antikörper zu produzieren und einen langfristigen Schutz gegen Pathogene bieten (71).

Gesteuert über die CXCR4-CXCL12-Achse migrieren sie in spezialisierte Überlebensnischen, die im Knochenmark, aber auch in der Lamina propria des Darms zu finden sind. Auch wenn diese Nischen bisher nicht ganz klar definiert sind, ist bekannt, dass sie dort über Zell-Zell-Kontakt zu Stroma-Zellen, Dendritischen Zellen und regulatorischen T-Zellen sowie über lösliche Faktoren wie die Zytokine IL-6 und APRIL und das Chemokin CXCL12 Überlebenssignale erhalten, die es den Zellen ermöglichen über Monate, Jahre oder sogar lebenslang zu überdauern (72, 73). Für die Maus konnte gezeigt werden, dass IgA- und IgG-exprimierende Plasmazellen sowohl im Knochenmark als auch im Darm nicht zirkulieren, sondern konstant in den jeweiligen Nischen verbleiben (74).

LLPCs sind nicht von Natur aus langlebig. Vielmehr hängt ihr Überleben entscheidend davon ab, ob sie eine der in ihrer Anzahl begrenzten Überlebensnischen im Knochenmark oder anderen sekundären Organen, sowie entzündeten Geweben erreichen und diese nutzen können (75-78).

Die LLPCs in den Nischen des Knochenmarks stellen die Hauptquelle des im Körper zirkulierenden IgGs dar (79), sind aber auch in der Lage, Antikörper vom IgA und IgM Isotyp zu produzieren (80, 81). LLPCs finden sich jedoch auch im Darm assoziierten lymphatischen Gewebe (GALT), wo 80% der humanen Plasmazellen lokalisiert sind und vorrangig IgA produzieren (82). GALT Überlebensnischen für Plasmazellen sind noch unzureichend charakterisiert, scheinen aber auch reich Überlebensfaktoren wie APRIL zu sein (83).

Weder die Abwesenheit von Antigenen, noch die Depletion von B-Zellen, beispielsweise im Rahmen von gegen CD20 gerichteten Immuntherapien, scheint die Produktion von Antikörpern durch die LLPCs zu beeinflussen (84, 85), daher kann davon ausgegangen werden, dass sie vollständig unabhängig von ausgereiften B-Zellen und B-Gedächtniszellen interagieren.

### 2.1.8 B-Gedächtniszellen

Neben der Generierung von Plasmablasten/-Zellen werden im Rahmen der GC-Reaktion B-Gedächtniszellen ausgebildet, die bei erneutem Antigenkontakt eine wesentlich schnellere und auch stärkere Antikörperantwort auslösen (55, 86).

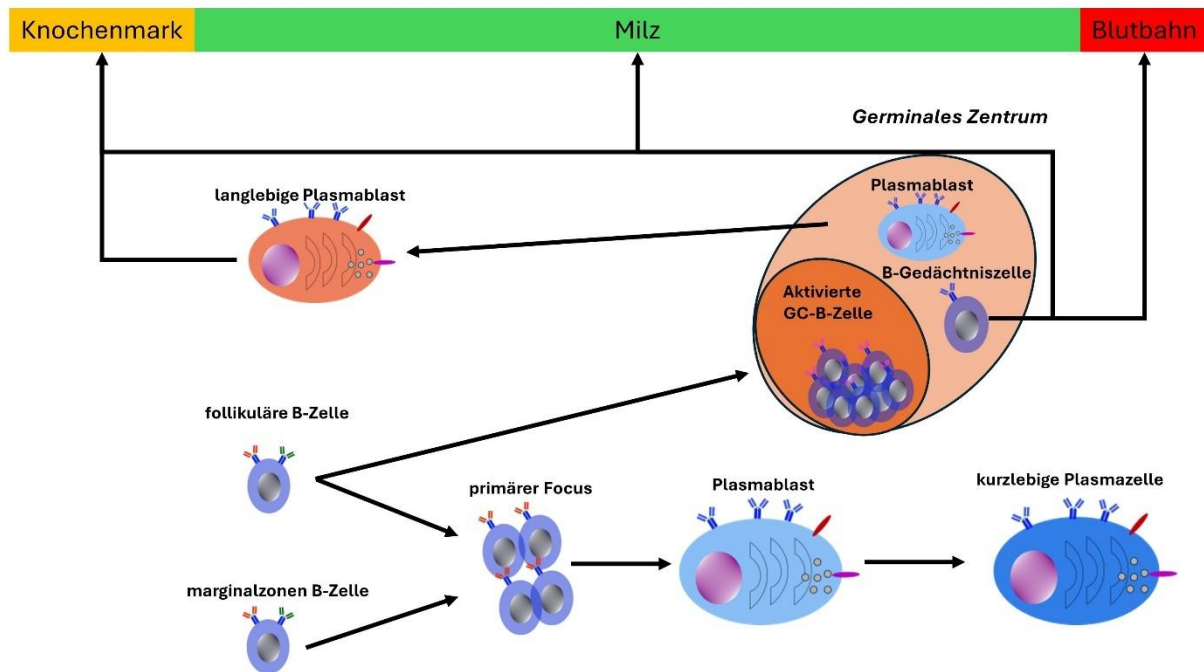
B-Gedächtniszellen zirkulieren beim Menschen im Blut (87, 88), ihre größten Reservoirare befinden sich jedoch in Knochenmark (89) und Milz (90).

Auch wenn murine und humane Gedächtniszellen mit TACI einen Rezeptor für BAFF und APRIL aufweisen und humane Gedächtniszellen zusätzlich noch höhere Level von BCMA und BAFF-R exprimieren (91-93), ist im Gegensatz zu den undifferenzierten B- und Plasmazellen für Gedächtniszellen keine Abhängigkeit von BAFF oder APRIL bekannt (91, 94).

Neuere Studien zeigen jedoch, dass im Mausmodell die Interaktion von BAFF und BAFF-R für das Überleben von B-Gedächtniszellen notwendig ist, sowie essentiell für die Bildung des GC-unabhängigen B-Gedächtniszell-Pools ist (95-97).

Die Hauptfaktoren für das Überleben von B-Gedächtniszellen scheinen jedoch zu sein, dass sie hohe Spiegel von Genen exprimieren, die an der Zellaktivierung, Costimulation und dem Überleben beteiligt sind (98-100), sie sich in einen Ruhezustand begeben können (101) und es zur mitunter zur Reaktivierung durch Antigen oder bystander-Signale kommt, die helfen die Überlebenssignale zu erneuern und damit die Langlebigkeit zu erhalten (102, 103).





**Abbildung 2** Überblick über die antigenabhängige B-Zellreifung (9, 47, 50, 51, 87, 88, 104-115)

## 2.1.9 Wichtige Transkriptionsfaktoren der B-Zell-Differenzierung

Während der B-Zellentwicklung und -differenzierung wirken eine Vielzahl verschiedener Transkriptionsfaktoren. Im Folgenden sind drei wichtige Faktoren näher beschrieben.

### 2.1.9.1 Paired box protein 5 (Pax-5)

Pax-5, auch als B-cell specific activator protein (BSAP) bezeichnet, ist ein entscheidender Transkriptionsfaktor in der B-Zell-Entwicklung. Es nimmt eine Schlüsselrolle bei der Festlegung der B-Zell-Linie ein, indem es Gene aktiviert, die für die Differenzierung zur B-Zelle notwendig sind, und gleichzeitig Gene unterdrückt, die nicht zur B-Linie gehören. Die Expression von Pax-5 beginnt in den frühen Stadien der B-Lymphopoese und wird durch den Early B-cell factor-1 (EBF-1) vermittelt (116-119). Pax-5 ist essentiell für die Weiterdifferenzierung der B-Zellen über das frühe Pro-B-Stadium hinaus und stellt die Aufrechterhaltung der B-Zell-Identität während der weiteren Differenzierung sicher (120).

Ein Ziel von Pax-5 sind unter anderem das Gen für CD19, eine Komponente des B-Zell-Korezeptors und das Gen für Igα, einer Signalkomponente sowohl des prä-B-Zell-Rezeptors als auch des B-Zell-Rezeptors. Fehlen Komponenten wie EBF oder Pax-5, kommt es zum Erliegen der B-Zell-Entwicklung (19, 121).

### **2.1.9.2 B-cell lymphoma 6 (Bcl-6)**

Bcl-6 spielt eine entscheidende Rolle für die B-Zellen in der Keimzentrumsreaktion in dem es dort als transkriptioneller Repressor wirkt (122, 123). Es wirkt negativ regulatorisch auf Gene, welche auf, während der somatischen Hypermutation und des Klassenwechsels entstehende, DNA-Schäden reagieren (124, 125).

### **2.1.9.3 B lymphocyte-induced maturation protein 1 (Blimp-1)**

Blimp-1 ist ein entscheidender Transkriptionsfaktor in der späten B-Zelldifferenzierung. Als sogenannter Masterregulator der Plasmazelldifferenzierung unterdrückt er zum einen Gene, die für die Aufrechterhaltung der B-Zell-Identität nötig sind, zum anderen fördert er die Expression von für die Plasmazellfunktion nötigen Genen (126). Blimp-1 induziert die Bildung von Plasmablasten und die Sekretion von Antikörpern, was für die effektive adaptive Immunantwort entscheidend ist (127).

Blimp-1 interagiert mit Pax-5 und unterdrückt dessen Expression. Somit wird die terminale Differenzierung der B- zur Plasmazelle ermöglicht. Umgekehrt kann Pax-5 aber auch Blimp-1 unterdrücken. Diese dynamische Wechselwirkung zwischen diesen beiden Transkriptionsfaktoren ist für ein Gleichgewicht zwischen B-Zell-Erhaltung und B-Zell-Differenzierung entscheidend (128-130).

Blimp-1 interagiert auch mit anderen Genen, wie Bcl-6, um die Zellproliferation zu reduzieren und die terminale Differenzierung als Effektorzelle zu fördern (126, 131).

### **2.1.10 Wichtige Zytokine der B-Zell-Differenzierung und Antikörperproduktion**

Im Rahmen der B-Zell-Entwicklung spielen viele Zytokine eine entscheidende Rolle, von denen im Folgenden die für die Differenzierung bedeutendsten besprochen werden.

#### **2.1.10.1 B cell activating factor belonging to the TNF family (BAFF)**

Das Zytokin BAFF wird durch das TNFSF13B-Gen kodiert und stellt einen zentralen Überlebens- aber auch Reifungsfaktor für B-Zellen dar (132-134). Das Überleben von unreifen B-Zellen im Knochenmark scheint unabhängig von BAFF zu sein, wohingegen es in BAFF-KO-Mäusen zu einem fast vollständigen Verlust von follikulären und Marginalzonen B-Zellen kommt (39, 135).

Ein besonderes Merkmal von BAFF ist seine Bindung an drei Rezeptoren: BAFF-R (spezifisch für BAFF), BCMA und TACI, die in unterschiedlichen B-Zell-Stadien exprimiert werden und zudem den Liganden APRIL binden (136, 137).

Gemeinsam mit APRIL trägt BAFF zum Überleben von LLPCs im Knochenmark bei (138).

#### **2.1.10.2 A proliferation-inducing ligand (APRIL)**

APRIL, ebenfalls ein Mitglied der TNF-Ligandenfamilie, spielt eine wesentliche Rolle im Überleben und der Differenzierung von B-Zellen. APRIL bindet an die Rezeptoren TACI und BCMA, die auf B-Zellen in späteren Entwicklungsstadien exprimiert werden (136, 139). Diese Interaktion fördert das Überleben von Plasmazellen und unterstützt die Erhaltung langlebiger Plasmazellen im Knochenmark (140), zusätzlich spielt es eine Rolle beim Immunglobulin-Klassenwechsel (141, 142). Die Expression von APRIL wird durch verschiedene Faktoren wie Zytokine (z.B. Interferon-alpha (IFN- $\alpha$ ) und Interferon-gamma (IFN- $\gamma$ ) reguliert (143) oder kann durch Entzündungsreaktionen getriggert werden (144).

#### **2.1.10.3 CD40 Ligand (CD40L)**

Der CD40L stellt ein weiteres für die B-Zell-Differenzierung zur Plasmazelle wichtiges Mitglied der TNF-Ligandenfamilie dar. Seine Interaktion mit seinem Bindungspartner CD40 spielt eine essenzielle Rolle innerhalb der T-Zell-abhängigen Antikörperantwort.

Die CD40L-vermittelte B-Zell-Hilfe fördert Proliferation, Bildung von GCs, Affinitätsreifung und den Immunglobulin-Klassenwechsel (145, 146). Nach Antigenkontakt internalisiert die B-Zelle den B-Zell-Rezeptor (BCR) mitsamt gebundenem Antigen, prozessiert es und präsentiert es über MHC-II an aktivierte T-Zellen. Die resultierende immunologische Synapse wird durch akzessorische Adhäsionsmoleküle stabilisiert und geht mit einer verstärkten Expression von CD40L und CD28 auf der T-Zelle einher (147, 148).

Auf der B Zelle führt die CD40-Ligation zu einer Hochregulation von CD80, CD86 und MHC-II, wodurch ihre Fähigkeit zur Antigenpräsentation und T-Zell-Aktivierung verstärkt wird (149).

#### **2.1.10.4 Interleukin 2 (IL-2)**

IL-2, ein Mitglied der Interleukin-2-Familie, steigert die Proliferation von mit CD40L stimulierten B-Zellen und kann in Kombination mit weiteren Zytokinen die Differenzierung aktivierter B-Zellen verbessern (150-155). Zusätzlich liefert IL-2 wichtige Überlebenssignale für B-Zellen in dem es die Bildung antiapoptotischer Proteine fördert (156).

#### **2.1.10.5 Interleukin 6 (IL-6)**

Ein weiteres Zytokin, IL-6, ein Mitglied der Interleukin-6-Familie, das im Rahmen der Fieberantwort als inflammatorischer Botenstoff bekannt ist (157), kann *in-vitro* das Überleben, sowie die Funktion von Plasmazellen fördern (158). Auch *in-vivo* fördert IL-6 die Aktivierung von B-Zellen und deren Differenzierung zu antikörperproduzierenden Plasmablasten (159), ebenso liefert es, ähnlich wie IL-2 Überlebenssignale in dem es die Expression antiapoptotischer Proteine fördert (72).

#### **2.1.10.6 Interleukin 10 (IL-10)**

IL-10 ein Mitglied der Interleukin-10 beziehungsweise Typ-II-Zytokinfamilie, ist ein pleiotropes, immunsuppressives Zytokin, das die Immunantwort moduliert und Interaktionen zwischen B-Zellen, T-Zellen und anderen Immunzellen vermittelt.

In B-Zellen wirkt IL-10 über alle Entwicklungsstadien hinweg proliferationsfördernd (160), vorausgesetzt, dass es parallel zu einer Interaktion von CD40 mit seinem Liganden kommt. Dieser Effekt wird durch IL-2 und IL-4 synergistisch verstärkt (161). Zudem fördert

IL-10 das Überleben von B-Zellen durch die Hochregulation antiapoptotischer Proteine wie BCL-2 (162, 163), sowie durch die Modulation der Telomerase-Aktivität (164). Sein Einfluss auf B-Zellen variiert je nach deren Aktivierungszustand und der Präsenz weiterer Zytokine (150, 165).

IL-10 unterstützt den Immunglobulinklassenwechsel zu IgG1 und IgG3, während in Anwesenheit von TGF- $\beta$  ein Wechsel zu IgA und IgE induziert wird (166).

#### **2.1.10.7 Interleukin 21 (IL-21)**

IL-21 ist, hinsichtlich der Proliferationsinduktion unter den derzeit identifizierte Zytokinen das potenteste, jedoch kann es ähnlich wie IL-10 nur in Kombination mit CD40L wirken (167, 168). Es induziert die Bildung des für Transkriptionsfaktors BCL6 (B cell lymphoma 6), einem Hauptregulator der GC-Reaktion, des für den Immunglobulin-Klassenwechsel essentiellen Enzyms AID, sowie von Blimp-1 dem Hauptregulator der terminalen B-Zell-Differenzierung (169-171). Hierdurch wird vor allem der Klassenwechsel hin zu IgG1, IgG3, IgA und IgE gefördert (172-175).

IL-21 ist sowohl am Differenzierungsprozess von Plasmazellen aus naiven B-Zellen, als auch aus B-Gedächtniszellen beteiligt (176). Bei der Plasmazellbildung aus GC-B-Zellen haben IL-10 und IL-21 redundante Funktionen, wirken aber zu verschiedenen Zeitpunkten der Reaktion unterschiedlich stark (177).

#### **2.1.10.8 Transforming Growth Factor-beta (TGF- $\beta$ )**

TGF- $\beta$  spielt eine entscheidende Rolle bei der Differenzierung von B-Zellen und der Klassenwechsel-Rekombination (CSR) zu IgA. TGF- $\beta$  fördert die Differenzierung von naiven B-Zellen zu IgA-produzierenden Plasmazellen, indem es die Expression von IgA-spezifischen Genen induziert (166, 178, 179).

Es wirkt durch die Aktivierung von Smad-Proteinen, die die Transkription von Zielgenen regulieren (179).

#### **2.1.10.9 Interferon-gamma (IFN- $\gamma$ )**

IFN- $\gamma$  wirkt als B-Zell-Wachstumsfaktor und fördert den Immunglobulin-Klassenwechsel zu IgG2a und IgG3 (180, 181). Gleichzeitig hemmt es die Produktion von IgG1 und IgE (174, 180).

### 2.1.11 Identifikation verschiedener B-Zell-Stadien anhand ihrer Oberflächenantigene

Verschiedene B-Zell-Stadien können bei Menschen anhand der Expression von verschiedenen Oberflächenantigenen identifiziert werden. Diese sind in **Tabelle 1** aufgelistet.

**Tabelle 1** Klassifizierung humaner B-Zell-Stadien anhand der Expression verschiedener Oberflächenproteine (9, 58, 182-201)

| Zelltyp            | Oberflächenproteine                                            |
|--------------------|----------------------------------------------------------------|
| Naive B-Zelle      | CD45, CD19, CD20, CD21, IgD, IgM <sup>low</sup> , MHCII        |
| GC-B-Zelle         | CD45, CD19, CD20, CD27, CD38, FAS                              |
| Plasmablasten      | CD45, CD19, CD20, CD27, CD38                                   |
| Plasmazelle        | CD45, CD138, CD38, CD19, CD27, PC-1, CXCR4, TACI, sIg          |
| LLPC               | CD27, CD38, CD138, BCMA, CXCR4                                 |
| B-Gedächtniszellen | CD45, MHCII, Immunglobulin, CD19, CD20, CD21, CD27, CD80, TACI |

## 2.2 Entwicklung des B-Zell-Systems des Huhns

Im Gegensatz zum Säuger findet der Hauptteil der B-Zell-Entwicklung beim Huhn nicht im Knochenmark, sondern in der, nur bei Vögeln vorhandenen, Bursa cloacalis, auch als Bursa Fabricius bezeichnet, statt.

Erstmals beschrieben wurde das Organ durch Girolamo Fabrizio da Acquapendente (Hieronymus Fabricius ab Aquapendente) in seinem posthum veröffentlichten Werk *De formatione Ovi et Pulli* und wurde zunächst als Speicherort für abgegebene Samenflüssigkeit fehlgedeutet (3, 202).

Die Rolle der Bursa Fabricius in der Antikörperentwicklung und als primäres lymphatisches Organ wurden erst wesentlich später erkannt (5, 6, 203).

Anhand ihrer Kompartimentierung lässt sich die B-Zell-Entwicklung beim Huhn in drei Phasen unterteilen: die prä-bursale, bursale und post-bursale Phase (**Abbildung 3**).

### 2.2.1 Prä-bursale und bursale B-Zell-Entwicklung

Ab Embryonaltag 5 wandern hämatopoetische Vorläuferzellen in verschiedene embryonale Gewebe (204) und beginnen dort mit der Umlagerung der Immunglobulin-Gene der leichten Kette (205). Zwischen Tag 8 und 14 migrieren die B-Zell-Vorläufer über die Blutbahn (206) in die Bursa (207), gesteuert durch den Chemokinrezeptor CXCR4 und seinen Liganden CXCL12 (208).

Nach erfolgreicher Gen-Umlagerung (209) wandern sie in die Anlagen der Bursafollikel (210) wo es zu einer exponentiellen Proliferation der Zellen kommt (206, 211). Die parallel induzierte Diversifizierung des BCRs und damit Diversifizierung des Repertoires an B-Zell-Spezifität erfolgt im Gegensatz zum Säuger nicht mittels Gene-Rearrangement sondern mittels Genkoversion (212-214).

Um die Bursa etwa ab dem Zeitraum des Schlupfes verlassen zu können benötigen die B-Zellen einen funktionellen BCR, ebenso müssen sie CXCR4 herunterregulieren (208, 215-218).

Insgesamt verlassen jedoch nur etwa 5% der in der Bursa gebildeten B-Zellen diese auch, die restlichen 95% gehen in-situ in Apoptose (219, 220).

Mit Eintritt in die Geschlechtsreife beginnt sich die Bursa zurückzubilden, bis sie letztlich vollständig verschwindet (221, 222). Während die B-Zellen beim Säuger über die gesamte Lebenszeit im Knochenmark nachgebildet werden, ist die B-Zell-Nachbildung beim Huhn in seinem primären B-Zell-Organ, der Bursa Fabricius, also auf eine relativ kurze Zeitspanne begrenzt.

### 2.2.2 Postbursale B-Zell-Entwicklung

Nach Verlassen der Bursa gelangen die sog. „recent bursal emigrants“ (RBEs), ausschließlich IgM exprimierenden B-Zellen (220), über das Blut in die Peripherie. Ihre Emigrationsrate beträgt etwa 1 % der peripheren B-Zellen pro Stunde (218).

Studien an bursektomierten Hühnern zeigen drei Haupt-B-Zell-Subpopulationen in der Peripherie:

1. Kurzlebige B-Zellen (LT2<sup>+</sup>, ~60 % der B-Zellen, Lebensdauer ~3 Tage) (218)
2. Langlebige B-Zellen (LT2<sup>-</sup>, IL12<sup>+</sup>, MHCII<sup>+++</sup>, Lebensdauer ~3 Wochen) (223)
3. Nicht-bursale B-Zellen (~5 % der peripheren B-Zellen), vermutlich aus post-bursalen Stammzellen (218, 224)

Da das Huhn keine Lymphknoten besitzt, besiedeln die post-bursalen, naiven B-Zellen lediglich die sekundäre lymphatische Organe, wie die Milz oder Schleimhautassoziierte lymphatisches Gewebe (MALT).

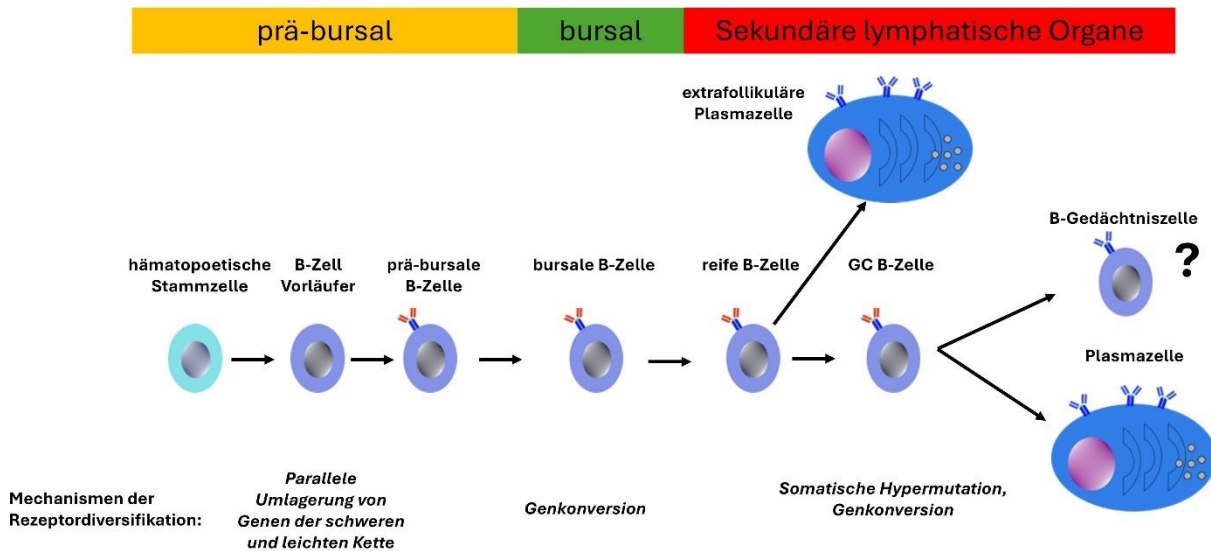
Im Gegensatz zum Säuger erfolgt bei den Hühner-B-Zellen keine Unterscheidung zwischen B1-, B2- oder Marginalzonen-B-Zellen und die B-Zellen exprimieren alle den Panleukozytenmarker CD45.

Die Aktivierung von naiven B-Zellen nach Kontakt mit Antigenen erfolgt vermutlich analog zum Säuger (4), auch bilden sich germinale Zentren, welche sich jedoch strukturell von denen der Säuger unterscheiden. Während die germinalen Zentren der Säuger eine klare Trennung von heller und dunkler Zone aufweisen, scheinen die GCs der Hühner homogener, jedoch konnte mittels BrdU (Bromdesoxyuridin) ein Ring proliferierender Zellen unterhalb der Kapsel nachgewiesen werden (4, 225). Dies deutet darauf hin, dass auch die GCs von Hühnern eine zonale Trennung aufweisen.

Die GCs des Huhns sind oligoklonal, entstammen also einer geringen Zahl aktivierter B-Zellen und der BCR wird mittels Genkonversion und somatischer Hypermutation



verändert (226, 227). Mittels immunhistochemische Verfahren konnte ein Immunglobulin-Klassenwechsel von IgM zu IgA und IgY nachgewiesen werden (225).



**Abbildung 3** B-Zell-Entwicklung des Huhns (4, 207, 212, 214, 225-236)

### 2.2.3 Plasmazellen

Ähnlich wie beim Säuger entstehen Plasmazellen nicht nur aus der GC-Reaktion sondern auch und können auch als Analoga der SLPCs aus einer extrafollikulären Reaktion entstehen (4, 236).

In Immunisierungsversuchen konnte gezeigt werden, dass die ersten IgM-positiven, antikörpersezernierenden Zellen (ASCs) nach etwa 48 Stunden in T-Zell-Bereichen der Milz entstehen, nach 72h findet man sie hauptsächlich in der roten Milzpulpa. Die ersten Ig-klassengewechselten Zellen findet man nach 96 Stunden. Sie exprimieren IgY, früher auch als IgG des Huhns bezeichnet.

Neben der Milz konnten Plasmazellen aller drei Immunglobulin-Isotypen des Huhns vor allem im MALT-Organen wie der Caecaltonsille, einer Ansammlung von dichtem lymphoretikulären Gewebe an der Basis der Caeca, oder der Harderschen Drüse, sowie der Lamina propria des Darms gefunden werden (236-239). Ob das Knochenmark des Huhns die beim Säuger beschriebenen Nischen enthält in denen Plasmazellen auf Grund verschiedener Faktoren längerfristig überleben können ist bislang nicht bekannt.

Jedoch gibt es im Vergleich zum Menschen (siehe **Tabelle 1**) bislang keinen plasmazellspezifischen Oberflächenmarker, welcher die Zellen mittels Durchflusszytometrie oder (immun-) histochemischen Methoden eindeutig identifizieren kann. Daher erfolgt die Identifizierung der Hühner-Plasmazellen bislang über deren klassische Morphologie, als große Lymphozyten mit einem dezentralen Nucleus der eine Wagenrad-Struktur aufweist und die große Mengen an Immunglobulin enthalten (4). Histologisch ist zudem der Verlust des Hühner-B-Zell-Markers chB6 beschrieben (240).

#### **2.2.4 B-Gedächtniszellen**

B-Gedächtniszellen konnten beim Huhn bislang nicht eindeutig identifiziert werden. Der einzige Nachweis für diesen Zelltyp ist bislang die erfolgreiche Induktion verstärkter Antikörper-Recall-Reaktionen nach Prime-Boost-Impfungen (241).

#### **2.2.5 Zytokine in der B-Zell-Differenzierung**

Von den beim Säuger beschriebenen Zytokinen sind im Huhn bislang nur die TNF-Familienmitglieder BAFF und CD40L mit ihrem Einfluss auf B-Zellen näher beschrieben (242-245). Weitere Zytokine die eine wichtige Rolle für die B-Zellen spielen, wie IL-7, IL-10 und IL-21 und ihre entsprechenden Rezeptoren wurden zwar beschrieben und kloniert, jedoch muss ihr genauer Einfluss auf die B-Zell-Differenzierung noch genauer untersucht werden (246-250)

##### **2.2.5.1 B cell activating factor belonging to the TNF family (BAFF)**

Im Hühnergenom zeigt BAFF eine hohe Sequenzhomologie zum Säuger, jedoch bestehen Unterschiede zwischen den BAFF-Systemen. Neben dem Nachweis von BAFF-mRNA in Geweben wie Bursa und Milz konnte BAFF auch in B-Zellen selbst nachgewiesen werden, was auf eine autokrine Produktion von BAFF durch die Zellen selbst hindeutet (244, 245, 251). Dieses Phänomen wurde bei Mensch und Maus bisher nur im Zusammenhang mit Autoimmunerkrankungen beschrieben wurde (252). Ein weiterer Unterschied zum Säuger ist, dass das Überleben der unreifen B-Zellen der Bursa im Gegensatz zu den unreifen B-Zellen im Knochenmark der Säuger (39, 135) von BAFF abhängig ist.

Neben dem BAFF-R liegt im Huhn noch TACI als weiterer Rezeptor für BAFF vor (253). Das Gen für BCMA, als möglichen weiteren Rezeptor für BAFF, konnte im Huhn zwar

identifiziert werden, liegt jedoch als Pseudogen vor (253, 254). APRIL, als weiterer Ligand für TACI, konnte im Hühnergenom bislang nicht nachgewiesen werden (4).

*In-vitro* verlängert rekombinantes BAFF das Überleben von Bursazellen, sowie von aus der Milz isolierten B-Zellen (245) ohne jedoch deren Proliferation zu fördern (243). *In-vivo* überexprimiertes BAFF führte zu einer deutlich gesteigerten Frequenz zirkulierender B-Zellen, wohingegen die Blockade von endogenem BAFF mit Hilfe eines humanen Rezeptors zu einer lange andauernden Depletion von B-Zellen führte (244).

Diese Ergebnisse zeigen die zentrale Rolle von BAFF nicht nur für das Überleben peripherer B-Zellen, sondern auch für die Entwicklung unreifer B-Zellen in der Bursa.

Nachdem BAFF im Gegensatz zum Säuger beim Huhn von B-Zellen selbst produziert wird (245), weist dies auf einen autokrinen Loop und damit grundsätzlich andere Regulationsmechanismen hin.

#### **2.2.5.2 CD40 Ligand (CD40L)**

Das CD40-CD40L-System wurde auch im Huhn identifiziert (255) und umfassend charakterisiert. So konnte gezeigt werden, dass die Zugabe von CD40L zu aus der Milz gewonnenen B-Zellen, eine Kultivierung über einen Zeitraum von bis zu drei Wochen ermöglicht. Zudem wurden die B-Lymphozyten zur Proliferation angeregt und regulierten den Pan-B-Zellmarker *chB6* herunter. Zeitgleich wurde ein Immunglobulin-Klassenwechsel von IgM zu IgY induziert, sowie die Immunglobulinproduktion (IgM und IgY) generell gesteigert (242).

Diese in Zellkultur gewonnenen Daten weisen auf eine größtenteils konservierte Funktion von CD40L und CD40 zwischen Säuger und Huhn hin (4) und zeigen, dass die Stimulation von Hühner-B-Zellen mittels CD40L diese Richtung eines Plasmablasten-Phänotyps differenziert.

### 2.2.6 Identifikation von B-Zellen anhand ihrer Oberflächenantigene

Die B-Zellen des Huhns in ihren verschiedenen Entwicklungsphasen sind über die im Folgenden aufgelisteten Antigene ansprechbar:

**Tabelle 2** Übersicht ansprechbar Oberflächenantigene auf Hühner-B-Zellen in verschiedenen Entwicklungsphasen nach Härtle et al. (256)

| Entwicklungsphase     | Antigene                                                                                                                                                               | Literatur                                 |
|-----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------|
| prä-bursale B-Zellen  | chB6 ( <i>ab ED10</i> ), BAFF-R ( <i>ab ED14</i> )<br>CXCR4/5 ( <i>in der Milz</i> ), Sialyl-Lewis <sup>x</sup><br>(CD15s)                                             | (208, 256-260)                            |
| bursale B-Zellen      | chB6, BAFF-R, MHCII, CD40, CXCR4,<br>CXCR5, <i>Wechsel von CD15s zu CD15</i>                                                                                           | (208, 217, 243,<br>255, 256, 258-<br>260) |
| emigrierende B-Zellen | Ig Light chain, MHCII, chL12, CXCR4 <sup>low</sup>                                                                                                                     | (208, 218, 256)                           |
| periphere B-Zellen    | CD5, chB6, BAFF-R, Ig Light chain, MHCII,<br>CD40                                                                                                                      | (242, 245, 255,<br>256, 258, 261)         |
| GC B-Zellen           | <i>keine spezifischen Marker vorhanden</i><br><i>möglicherweise CD57</i>                                                                                               | (256, 262)                                |
| B-Gedächtniszellen    | <i>keine spezifischen Marker vorhanden</i>                                                                                                                             | (256)                                     |
| Plasmazellen          | <i>keine spezifischen Marker vorhanden</i><br><i>keine bis wenig Expression von chB6</i><br><i>möglicherweise Expression von CD57</i><br>intrazelluläres Immunglobulin | (240, 242, 256,<br>262)                   |

## **2.3 Induktion und Verteilung der verschiedenen Immunglobulin-Isotypen**

Immunglobuline, auch als Antikörper bezeichnet, sind eine Gruppe spezialisierter Proteine, die von B-Zellen produziert werden und eine zentrale Funktion im Immunsystem aller Vertebraten ab den Fischen erfüllen.

### **2.3.1 Immunglobulinklassen der Säuger**

Beim Säuger existieren fünf Hauptklassen oder Isotypen von Immunglobulinen: IgM, IgG, IgA, IgE und IgD.

Naive B-Zellen exprimieren Immunglobuline der Isotypen IgM und IgD in ihrer membrangebundenen Form als B-Zellrezeptor auf ihrer Zelloberfläche. Nach Antigenkontakt differenzieren sich diese Zellen zu Plasmablasten bzw. Plasmazellen und initiieren die humorale Immunantwort, in der sie zunächst IgM-Antikörper sezernieren. In seiner monomeren Form weist IgM eine vergleichsweise geringe Bindungsaffinität auf, liegt jedoch meist als Pentamer vor, wodurch die höhere Anzahl an Bindestellen eine gesteigerte Avidität ermöglicht und so die schwächere Affinität der einzelnen Monomere kompensiert. Aufgrund der molekularen Größe des Pentamers zirkulieren sie hauptsächlich im Blut und diffundieren weniger in den Interzellularraum, da die Größe des Moleküls das Verlassen der Blutgefäße behindert. IgM wird sowohl von B1, B2 als auch Marginalzonen-B-Zellen produziert.

IgD, das zweite Immunglobulin der naiven B-Zelle, ist in löslicher Form nur in geringen Mengen im Serum vorhanden. Seine genaue Funktion ist nicht vollständig geklärt, es scheint jedoch in der Zellmembran für die Modulation der B-Zellaktivierung verantwortlich zu sein (263).

Im Rahmen der Immunantwort erfolgt ein Klassenwechsel von IgM zu den anderen Immunglobulinisotypen, IgG, IgA und IgE. Diese liegen als Mono- oder Dimere vor, sind also wesentlich kleiner als das pentamere IgM und können so leichter vom Blut in die Gewebe diffundieren.

IgG stellt die Hauptklasse von Immunglobulin im menschlichen Blut dar und kann beim Menschen in vier Subklassen, IgG1-4, unterteilt werden (264). Als einziges Immunglobulin kann es die humane Placenta passieren und so Neugeborenen einen passiven

Immunschutz gewähren. Seine Hauptaufgabe besteht darin, Antigene für Phagozyten zu opsonieren und das Komplementsystem zu aktivieren. Es wirkt hauptsächlich im Gewebe. Der Klassenwechsel von IgM zu IgG wird unter anderem von den Zytokinen IL-10, IL-21 und IFN- $\gamma$  induziert.

IgA ist das dominante Immunglobulin auf mukosalen Oberflächen, auf denen seine Hauptaufgabe darin besteht, Antigene zu neutralisieren. Seine Wirkungen als Opsonin und in der Aktivierung des Komplementsystems sind wesentlich schwächer als die von IgG. Es liegt beim Menschen in zwei Isoformen, IgA1 und IgA2 vor, wobei IgA1 hauptsächlich als Monomer im Blut zirkuliert und IgA2 hauptsächlich in seiner dimeren Form auf mukosalen Oberflächen im Darm vorkommt (265-269). Im Bereich des Kopfes wird IgA sowohl im Nasen-assoziierten lymphatischen Gewebe (NALT, nasal associated lymphatic tissue) als auch Augen-assoziiertem lymphatischen Gewebe (EALT, eye associated lymphatic tissue) sekretiert und sorgt dort lokal für den Schutz vor Infektionen (270-272). Für den Klassenwechsel zu IgA ist vor allem TGF- $\beta$  verantwortlich.

IgE kommt nur zu sehr geringen Mengen in Blut oder extrazellulären Flüssigkeiten vor. Es findet sich hauptsächlich an Mastzellen gebunden vor, die unter der Haut, der Mukosa oder entlang von Blutgefäßen liegen. Die Bindung von Antigen an das mastzellassoziierte IgE befähigt die Zellen, Entzündungsmediatoren und Chemoattraktanten freizusetzen, welche eosinophile und basophile Granulozyten anlocken. IgE-Immunantworten spielen vor allem bei parasitären Infektionen und allergischen Reaktionen eine Rolle (273, 274). Der Klassenwechsel zu IgE wird unter anderem von IL-10 und IL-21 induziert.

Hinsichtlich der Menge an produziertem Immunglobulin zeigt IgA mit 60-66 mg/kg Körpergewicht pro Tag die höchste Biosyntheserate, gefolgt von IgG mit 34mg, IgM mit 8mg, IgD mit 0,4mg und IgE mit 0,0016mg. Bei der Verteilung im menschlichen Serum dominiert IgG mit etwa 85%, gefolgt von IgA mit 10-15% und IgM mit etwa 5%. Die niedrigsten Anteile haben IgD und IgE mit 0,3% beziehungsweise 0,02% (269, 275-282).

### 2.3.2 Immunglobulinklassen des Huhns

Im Gegensatz zu Säugetieren finden sich im Huhn nur drei Immunglobulinklassen: IgM, IgY und IgA.

Wie auch beim Säugetier ist IgM das Immunglobulin der naiven, nicht-klassengewechselten B-Zellen und zeigt sich sowohl strukturell als auch funktionell homolog. Nach der ersten Exposition gegenüber einem neuen Antigen ist IgM der vorherrschende Isotyp, jedoch ist die IgM-Antikörperantwort, wie beim Säuger, in der Regel nur temporär, bevor es im Rahmen der GC-Reaktion zur Bildung und spezifischer, klassengewechselter Antikörper kommt (283, 284).

Ein strukturelles Analogon zu IgD konnte beim Huhn bislang nicht nachgewiesen werden, jedoch ist es beim Strauß und einigen Neoaves beschrieben (285)

IgY ist die dominante Immunglobulinklasse im Serum der Hühner, die nach IgM in der primären Antikörperreaktion gebildet wird. Darüber hinaus stellt es den Haupt-Isotyp in der sekundären systemischen Antikörperreaktion dar. Es zeigt sowohl Homologien zu IgG als auch zu IgE des Säugers, entspricht aber funktionell IgG (286, 287).

IgA hingegen ist das dominante Immunglobulin in auf mukosalen Oberflächen und liegt neben der monomeren Form, anstatt wie die dimere Form bei Säugetieren, als Trimer oder Tetramer vor (288, 289).

Hinsichtlich der Verteilung im Serum ist IgY mit 5-7 mg/ml, ähnlich wie das IgG des Säugers. das dominante Immunglobulin, gefolgt von IgM mit 1-2 mg/ml und IgA mit 0,35–0,65 mg/ml, jedoch ist nicht bekannt, wo die Zellen lokalisiert sind, die diese großen Mengen an IgY sezernieren (288-292).

### 3 Zielsetzung

Zwar sind grundsätzliche Mechanismen der finalen Differenzierungsschritte von B- zur antikörpersezernierenden Plasmazelle, wie zum Beispiel die Bildung germinaler Zentren oder Immunglobulin-Klassenwechsel bekannt, jedoch sind die genauen funktionellen Mechanismen innerhalb dieser Zellen bislang weitgehend unbekannt. Ebenso fehlt es zum Teil noch an Wissen über die genaue Lokalisation der Zellen, so zum Beispiel ist nach wie vor nicht bekannt, wo sich die Zellen befinden, die für die hohen IgY-Antikörperspiegel im Serum der Hühner verantwortlich sind.

Um diese Fragen zu adressieren, mangelt es jedoch bislang an plasmazellspezifischen Markern anhand derer die Zellen gezielt isoliert und im Anschluss genauer charakterisieren werden könnten.

Ziel dieser Arbeit war es, eine Grundlage zu schaffen, die es ermöglicht, Plasmazellen und die in ihnen ablaufenden molekularen Prozesse zukünftig näher untersuchen zu können.

Hierfür wurden sowohl *in-vitro* als auch *ex-vivo* Ansätze gewählt.

*Das Ziel des in-vitro Ansatzes war es Plasmazellen aus Milzleukozyten zu generieren und Veränderungen der Expression von Oberflächenantigenen sowie die Änderung im Transkriptionsfaktorprofil genauer zu untersuchen.*

*Ex-vivo sollte die Verteilung von Zellen mit einem vermuteten Plasmazellphänotyp in den Leukozytenpopulationen verschiedener Organe, sowie der Einfluss von Impfung und Alter untersucht werden. Diese Daten wurden gemeinsam mit dem in-vitro-Ansatz am 21.11.2024 in *Frontiers in Immunology* veröffentlicht (**Publikation: Characterization of Class-Switched B cells in Chickens**).*

In einem gesonderten *ex-vivo* Ansatz wurde die Harderschen Drüse untersucht, ein Organ, aus dem von histologischen Untersuchungen bekannt ist, dass es viele Plasmazellen enthält. Ziel war es anhand verschiedener Oberflächenmarker ein Isolationsprotokoll zu entwickeln, um Plasmazellen zukünftig detaillierter untersuchen zu können. Die Ergebnisse dieser Untersuchungen sind unter „**unveröffentlichte Daten**“ dieser Arbeit in Form eines Manuskripts beigelegt.



## 4 Publikation: Characterization of Class-Switched B cells in Chickens

### 4.1 Publikation

Die Publikation “*Characterization of class-switched B cells in Chickens*” erschien am 21.11.2024 in Frontiers in Immunology, Section B cell Biology, Volume 15 – 2024, <https://doi.org/10.3389/fimmu.2024.1484288>.

Diese Publikation beschreibt im Wesentlichen den *in-vitro* Ansatz zur Generierung von Plasmazellen, sowie grundlegende Daten zu Verteilung von Immunglobulin-positiven Zellen in Organen, sowie den Einfluss von Alter und Impfung.

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# Characterization of class-switched B cells in chickens

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While B cell development in the birds' primary B cell organ, the bursa Fabricius, is relatively well understood, very little is known about post bursal B cell differentiation into plasma and memory cells though these cells are essential for a protecting antibody response and so far, no specific markers for these cells were available. Since immunoglobulin class switch is one part of the B cell differentiation process, our objective was to conduct a first detailed investigation of class-switched chicken B cells. As only very few IgY and IgA expressing cells were detected in lymphoid organs of young chickens, we used CD40L and IL-10 to establish a prolonged *in vitro* culture system, which induces B cell proliferation, class switch to IgY and IgA and enhanced antibody secretion. This enabled a phenotypic analysis of differentiating B cells. Importantly, these cells lost surface expression of the B cell markers *chB6* and *BAFF-R*. B cell receptor surface expression remained unchanged, showing that while differentiating toward plasma cells, B cells can be addressed by L chain staining. Newly generated potential plasma cell markers *CD138* and *TACI* showed only a transient expression on cultured cells and rather act as markers for B cell activation than plasma/memory cells in general. *CD57* upregulation was connected to activation and blast formation but not to class switch. We also examined potential changes in class-switched cells in different age groups and post vaccination. Surprisingly, bursa involution, laying and age had no distinct effects on the presence of class-switched cells, but we detected significantly more class-switched B cells post vaccination. Hence, we are now able to generate class-switched plasmablasts *in vitro* for a more detailed characterization and can address them under different conditions in chickens for further analysis of their B cell response.

## KEYWORDS

chicken, B cell differentiation, Ig class switch, CD40L, IL-10, vaccination

## 1 Introduction

Vaccination is an essential measure to protect commercial poultry flocks against a multitude of pathogens. The mode of action of many vaccines is the induction of a protective antibody response (1, 2). Hence, to improve vaccine design and application, a better knowledge on antibody secreting cells (ASCs) and B cell memory in the chicken is indispensable. As the chickens B cell system differs substantially from that in human and mice, knowledge from these species can be transferred only to a very limited extent. The most striking difference is the bursa of Fabricius, a gut associated lymphoid tissue, which serves as the avian primary B cell organ (3). In addition, chickens have no lymph nodes, which strongly limits the number of secondary lymphoid tissues and the potential locations for B cell – T cell-contact. In the spleen, no separation between marginal and follicular B cells is visible and the differently structured germinal centers (GC) are located in the T cell area (4). Furthermore, the bursa involutes with sexual maturity, ceasing the production of new B cells in the primary B cell organ (5). Consequently, the maintenance of the peripheral B cell pool is left to a largely unknown mechanism (6, 7).

Whereas the processes of B cell receptor (BCR) repertoire generation in the bursa are relatively well understood (8), there is still only scarce information on the differentiation processes happening in the periphery after the cells have left the bursa. Chicken plasma cells, as finally differentiated ASCs, have been identified by immunohistochemistry on the basis of their typical morphology as large cells with an eccentric nucleus with cartwheel structure and a strong intracellular staining for immunoglobulins (8). But only very few reports exist about their differentiation from naïve B cells (9) and their isolation (10) for a more detailed characterization. For chicken B memory cells, the only prove of their existence so far is the successful induction of enhanced antibody recall reactions by prime - boost vaccination (11) and immunoglobulin class switching.

The phenotype of mammalian memory and plasma cells can be addressed by various markers like CD27 for memory cells (12) and CD138 for plasma cells (13, 14). In addition, during the differentiation process a switch in the expression of the three receptors for B cell activating factor of the TNF family (BAFF) occurs: while immature and mature B cells express BAFF-R, activation and differentiation induces a switch to the expression of transmembrane activator and calcium-modulator and cytophilin ligand interactor (TACI) (15–17) and plasma cells additionally express the B cell maturation antigen (BCMA) (18, 19).

Before chicken B cell precursors migrate to the bursa, they start to express the chB6 antigen, a highly glycosylated type I transmembrane protein (20). Subsequently, both bursal and peripheral chicken B cells can be addressed by their expression of chB6 (21, 22). However, plasma cells have been shown by immunohistochemistry to lose the chB6 antigen to an undetectable level (21), an observation which has been confirmed by *in vitro* induced plasmablast like cells, which had a strongly reduced chB6 expression (23). Bursal and peripheral chicken B cells express BAFF-R (24–26), which can be addressed by a specific antibody (27). A chicken homologue of TACI was also identified

(24), but surface expression of TACI protein was not yet analyzed. Though a chicken BCMA gene was identified, it was shown to be a pseudogene (24, 28). Hence, if a differentiation-stage specific switch in the expression of BAFF receptors occurs on chicken B cells, it must follow a different pattern than that in mammals, due to the expression of only two receptors.

Initially, all naïve B cells express an IgM type BCR. One of the events, which can occur during differentiation of a B cell in the GC is immunoglobulin (Ig) class switch from IgM to other Ig isotypes (29). Class-switched B cells can therefore be regarded as differentiated cells. In chickens the repertoire Ig isotypes is limited (30–32) and class switch can only occur from IgM toward IgY, described as intermediate between IgG and IgE (31, 33) or to IgA, the dominant Ig of mucosal surfaces (34–36).

In contrast to human blood, where age dependent up to 25% of blood B cells have undergone class switch (37–39), the vast majority of peripheral chicken B cells was reported to express IgM (40, 41), making other isotypes infrequent. For a better access to these rare cells, an *in vitro* culture system could be helpful.

B cell activation includes two main signals: antigen binding to the BCR and the binding of CD40 by CD40L, which is provided by activated T helper cells (42). Fine adjustment of the reaction is obtained by the response to additional cytokines like IL-4, IL-6, IL-10 and IL-21 (43–48) which direct B cell fate toward memory or plasma cells and the type of class switch. *In vitro*, strong CD40-signaling induced by recombinant CD40L can be sufficient for B cell activation (23, 49). In cultures of human B cells, addition of IL-10 to CD40L activated B cells leads, depending on the duration and sequence of stimulation, to the differentiation into a memory or plasma cell phenotype (46) and induces class switch to IgG (50).

Using cultures of chicken B cells, we have shown that recombinant CD40L drives them toward a plasma cell phenotype and enhances antibody production (23). The availability of recombinant chicken IL-10 (51) prompted us to test its potential for B cell differentiation *in vitro*.

We established and characterized a culture system which enables Ig class switch and antibody secretion. Based on the gained knowledge we have examined the presence of class-switched B cells pre- and post-bursal involution and post IBDV vaccination.

## 2 Materials and methods

### 2.1 Animals

Fertilized eggs of M11 white layer chicken (B2/B2 haplotype) were provided by Prof. Dr. Steffen Weigend (FLI Mariensee) and incubated and hatched at the Faculty of Veterinary Medicine, LMU Munich. Animals did not receive vaccinations except if required for experiments. The animals were housed in groups under conventional conditions and received both food and water *ad libitum*. Chickens of the layer-line Lohmann-Sandy were kept under conventional conditions on different organic farms and vaccinated following standard vaccination protocols for poultry. Experiments with M11 birds were performed at the age of 16–24



weeks, animals of the Lohmann-Sandy chicken were 7, 17, 27 and 70 weeks old.

For the IBDV vaccination experiment 6-week-old M11 chickens were vaccinated orally with one dose of Nobilis® Gumboro 228E (Intervet Germany GmbH, Unterschleißheim, Germany), as defined by the manufacturer.

## 2.2 Cell preparation

The animals were euthanized through exsanguination following anesthesia. Organs were aseptically dissected and placed in transport media (RPMI 1640, Thermo Fisher Scientific, Waltham, USA) supplemented with 1% Penicillin-Streptomycin (Merck KGaA, Darmstadt, Germany) at room temperature (RT) until further processing.

Heparinized blood was collected from the jugular vein, bone marrow (BM) was acquired by flushing it from the femoral bone. Single cell suspensions of BM, spleen and caecal tonsil were obtained by mechanical dissociation of the tissue through a stainless-steel. Subsequently, leukocytes were separated using density gradient centrifugation with Histopaque®-1077 (Merck KGaA, Darmstadt, Germany). All steps involving tissue preparation and centrifugation were conducted at room temperature to maintain optimal cellular viability and integrity for chicken B-cells.

## 2.3 Cell culture and stimulation

Splenocytes were cultured in 24-well-plates with  $2.5 \times 10^6$  c/ml in IMDM (Thermo Fisher Scientific, Waltham, USA) supplemented with 8% FBS Superior (Merck KGaA, Darmstadt, Germany), 2% chSerum ((Thermo Fisher Scientific, Waltham, USA) and 1% Penicillin-Streptomycin (Merck KGaA, Darmstadt, Germany) at 40°C with 5% CO<sub>2</sub>. Cell culture supernatant produced by a bioreactor containing soluble recombinant cytokines (CD40L and

IL-10) were added at a concentration of 2.5%. To maintain optimal cell growth and viability half of the medium was replaced every second day and cells were split into two wells at the third day of culture.

## 2.4 Recombinant cytokines

HEK293 cells were stably transfected with a trimerized chCD40L-muCD8 construct that has been previously described (23, 52). Clones exhibiting the highest production rates were selected for further use and cultured in a WHEATON® CELLLine™ bioreactor (DKW Life Sciences, Wertheim, Germany) for adherent cells with RPMI (Thermo Fisher Scientific, Waltham, USA), supplemented with 20% FBS (Merck KGaA, Darmstadt, Germany). Bioreactor supernatant was sterile filtered and titrated to determine the optimal concentration.

For rchIL10, a pCI-neo-chIL-10 expression construct was generously supplied by Dr. Pete Kaiser (Roslin Institute, Edinburgh, UK) (51). Subsequently, the chIL10 sequence was cloned into a modified pCR3 vector containing a flag-sequence (kindly provided by Dr. Pascal Schneider, Lausanne, Switzerland) by using primers (Table 1) to introduce PstI and EcoRI restriction sites. The resultant chIL10-Flag construct was then stably transfected in HEK293 cells, and the clone demonstrating the highest production rate was subsequently cultured under selective conditions in a WHEATON® CELLLine™ 1000AD bioreactor (DKW Life Sciences, Wertheim, Germany) with RPMI (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% FBS (Merck KGaA, Darmstadt, Germany) and 250 µg/ml Gentamicin (G418) (Carl Roth GmbH + Co.KG, Karlsruhe, Germany). To obtain purified rChIL-10, cell culture supernatants were purified by affinity chromatography using an anti-Flag-M2 column (Merck KGaA, Darmstadt, Germany). After initial experiments with purified protein, cell culture supernatants were titrated against the purified protein to determine the optimal concentration and used for further experiments.

TABLE 1 Forward and reverse primers used for qRT-PCR.

| Gen            | Forward Primer                                | Reverse Primer                                 |
|----------------|-----------------------------------------------|------------------------------------------------|
| <i>chIL10</i>  | 5'ATATCTGCAGTGCACCTGCCTGCCA <sup>3'</sup>     | 5'GCGCGAATTCTCACTTCCTCCTCCTC <sup>3'</sup>     |
| <i>chCD138</i> | 5'TAGAATTCAACCTTCTCTCTGAAGATCTC <sup>3'</sup> | 5'TACTCGAGTTATGCATAGAAGCTCTCTCTG <sup>3'</sup> |
| <i>chTACI</i>  | 5'ATGGACGGGACGTGCCTGGGAAGAGC <sup>3'</sup>    | 5'GCACCATGGGCGTCTTCTCTTGC <sup>3'</sup>        |
| <i>RPL13</i>   | 5'GAGGTGCCGACTGTCAGAT <sup>3'</sup>           | 5'ATCGTCCGAGCAAACTTTTGT <sup>3'</sup>          |
| <i>chB6</i>    | 5'GATCGCTGCCCTCCAA <sup>3'</sup>              | 5'TGGCTTCCACGTCAGCTATC <sup>3'</sup>           |
| <i>CD79b</i>   | 5'GCGTCCCCATGCTCCTCTTCTTA <sup>3'</sup>       | 5'GCAGCACCCTCACTCCTCTCCT <sup>3'</sup>         |
| <i>PAX5</i>    | 5'CCAGCAGCAGTTGGAAGTGT <sup>3'</sup>          | 5'CTCTGGTTTGTATGGGCTCTGT <sup>3'</sup>         |
| <i>µS</i>      | 5'GGAGAACCCCGAAATGAGT <sup>3'</sup>           | 5'GCCAACCAAGGAGACATT <sup>3'</sup>             |
| <i>BLIMP1</i>  | 5'GTGGTATTGCCGAGACTTTC <sup>3'</sup>          | 5'GGGTTTGTGTGAGGTTCATATT <sup>3'</sup>         |
| <i>BCL6</i>    | 5'GAAGACCCCAAGGAAGAGTT <sup>3'</sup>          | 5'CTGAGACATCTCTGCCCTCGATAAG <sup>3'</sup>      |
| <i>AICDA</i>   | 5'CGTCTGAAACCCAGCAAGAGT <sup>3'</sup>         | 5'TGTCCATGTCAGCTGGGTCT <sup>3'</sup>           |

## 2.5 [<sup>3</sup>H]-Thymidine assay

To detect cell proliferation,  $1 \times 10^6$  isolated spleen leukocytes were cultured in 96-well-plates in the presence and absence of CD40L, IL-10 and the combination of both cytokines at 40°C with 5% CO<sub>2</sub>. Medium served as control. After 48 hours cells were pulsed with [<sup>3</sup>H]-Thymidine (GE HealthCare, Amersham, UK) and harvested 16 hours later.

## 2.6 mAbs for chCD138 and chTACI

For the generation of an anti-CD138 (Syndecan 1) antibody, we amplified the chCD138 full length sequence (ENSGALT00010019066.1) from Harderian gland cDNA (see Table 1 for primers). To generate an anti-chTACI mAb, we amplified the full length sequence from pcTACI, a construct of chTACI in pcneo, kindly provided by Dr. John Young (24) (Institute for Animal Health, Compton, UK) (see Table 1 for primers). The PCR products were ligated into pcDNA 3.1/CT-GFP-TOPO (Thermo Fisher Scientific, Waltham, USA). Successful cloning was confirmed by restriction enzyme digest and subsequent Sanger

sequencing. HEK293 cells were stably transfected and selected for green fluorescent protein (GFP) fluorescence. BALB/c mice were immunized three times with  $1 \times 10^8$  HEK293-chCD138-GFP or HEK293-chTACI-GFP.

Murine spleen cells were fused to SP2/0-Ag14 cells, and supernatants of resulting hybridomas were tested by flow cytometry on transfected and untransfected HEK293 cells. The selected hybridoma 7G10 for chCD138 is an IgG1, the selected hybridoma 1H4 for chTACI an IgG2b antibody.

## 2.7 Flow cytometry, cell sorting and cell counting

Staining of cells for flow cytometric analysis was performed according to standard procedures. Briefly,  $10^6$  cells were transferred to a 96-well plate and incubated with Fixable Viability Dye eFluor™ 780 dilution (Thermo Fisher Scientific, Waltham, USA). Subsequently, cells were stained with 50 µl of monoclonal antibody (mAb) mixture (see Table 2). mAbs were either directly conjugated or detected by fluorochrome conjugated secondary antibodies (Table 3).

TABLE 2 Primary mAbs and directly conjugated mAbs.

| Antigen         | Clone | Species | Isotype | Fluorochrome | Source of antibody      |
|-----------------|-------|---------|---------|--------------|-------------------------|
| chB6            | AV20  | Mouse   | IgG1    |              | Southern Biotech        |
| chB6            | AV20  | Mouse   | IgG1    | Pacific blue | Southern Biotech        |
| chB6            | AV20  | Mouse   | IgG1    | RPE          | Southern Biotech        |
| chBAFF-receptor | 2C4   | Mouse   | IgG1    |              | (27)                    |
| chCD138         | 7G10  | Mouse   | IgG1    |              | this manuscript         |
| chCD25          | 28-4  | Mouse   | IgG3    |              | (118)                   |
| chCD28          | 2-4   | Mouse   | IgG2a   |              | (119)                   |
| chCD4           | CT4   | Mouse   | IgG1    | FITC         | Southern Biotech        |
| chCD8           | 3-298 | Mouse   | IgG2b   |              | (120)                   |
| chCD80          | AV82  | Mouse   | IgG2a   |              | The Pirbright Institute |
| chCXCR4         | 9D9   | Mouse   | IgG2a   |              | (121)                   |
| chCXCR5         | 6A9   | Mouse   | IgG1    |              | (122)                   |
| chMHCII         | 2G11  | Mouse   | IgG1    |              | Southern Biotech        |
| chTACI          | 1H4   | Mouse   | IgG2b   |              | this manuscript         |
| Human CD57      | NK-1  | Mouse   | IgM     | APC          | BD-Bioscience           |
| chL chain       | 2G1   | Mouse   | IgG1    | CF405M       | Bio-Rad                 |
| IgA             | A1    | Mouse   | IgG2b   |              | Southern Biotech        |
| IgA             | K3    | Mouse   | IgG2a   |              | (123)                   |
| IgA             | K3    | Mouse   | IgG2a   | Biotin       | (123)                   |
| IgM             | M1    | Mouse   | IgG2b   |              | Southern Biotech        |
| IgM             | M1    | Mouse   | IgG2b   | Biotin       | Southern Biotech        |
| IgY             | G1    | Mouse   | IgG1    |              | Southern Biotech        |
| IgY             | 4D12  | Mouse   | IgG1    | Biotin       | (123)                   |
| TCRγδ           | TCR1  | Mouse   | IgG1    |              | Southern Biotech        |



TABLE 3 Fluorochrome conjugated secondary antibodies.

| Antigen      | Fluorochrome    | Source of antibody       |
|--------------|-----------------|--------------------------|
| Mouse-IgG1   | APC             | Jackson Laboratories     |
| Mouse-IgG1   | FITC            | Southern Biotech         |
| Mouse-IgG1   | BV421           | Jackson Laboratories     |
| Mouse-IgG2a  | FITC            | Southern Biotech         |
| Mouse-IgG2a  | Alexa Fluor 647 | Thermo Fisher scientific |
| Mouse-IgG2b  | RPE             | Southern Biotech         |
| Mouse-IgG3   | FITC            | Southern Biotech         |
| Mouse-IgG3   | Alexa Fluor 647 | Southern Biotech         |
| Streptavidin | APC             | Jackson Laboratories     |

If necessary, when the unconjugated primary antibodies and the direct conjugates were of the same isotype, a blocking step with normal mouse serum (Jackson ImmunoResearch, West Grove, USA) was interposed before applying the directly conjugated antibody.

All incubation steps were carried out for 20 minutes in the dark on ice. In between the incubation steps, cells were washed with 200 µl of staining buffer (PBS pH 7.2 (Thermo Fisher Scientific, Waltham, USA), 1% bovine serum albumin and 0.1% NaN<sub>3</sub> (ITW Reagents, Castellar del Vallès, Spain)).

The anti-chicken L chain mAb (Table 2) was conjugated to CF405M using the Mix-n-Stain™ CF™ 405M Antibody Labeling Kit (Merck KGaA, Darmstadt, Germany).

For optimal staining results, all used mAbs and fluorochrome-conjugated secondary antibodies were titrated to their optimal dilution prior to the experiments.

For analysis of different tissues, 100.000 viable CD45<sup>+</sup> single cells were recorded per sample, for analysis of *in vitro* cultures, 50.000 viable single cells.

Flow cytometry was performed with a FACSCanto (Becton Dickinson, Heidelberg, Germany) and data were analyzed using FACSDiva (Becton Dickinson, Heidelberg, Germany) and FlowJo (FlowJo LLC, Oregon, USA) software, version 10.10.0.

Sort purification of cells was performed on a FACSARIA Illu (Becton Dickinson, Heidelberg, Germany) with FACS DIVA software.

The number of living cells was determined by trypan blue exclusion, and the frequency of living cells, negative for Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific, Waltham, USA), was analyzed by flow cytometry as described above. Absolute numbers of viable cells were calculated from these values.

## 2.8 qRT-PCR

For qRT-PCR, RNA of sort purified L chain positive/CD80 positive and L chain positive/CD80 negative splenic leukocytes, was prepared using the ReliaPrep™ RNA Miniprep Systems kit (Promega, Madison, USA) following the manufacturer's instructions.

The RNA was quantified on a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA quality was

determined on a 2100 Bioanalyzer (RIN > 9) (Agilent Technologies, Santa Clara, CA, USA). cDNA was prepared using the GoScript™ Reverse Transcriptase kit (Promega, Madison, USA) according to manufacturer's instruction and analyzed for the relative abundance of *RPL13*, *chB6*, *CD79b*, *µS*, *BCL6*, *PAX5*, *BLIMP1* and *AICDA* using primers from Eurofins Genomics Germany GmbH (Ebersberg, Germany) specified in Table 1 and the GoTaq® qPCR MasterMix (Promega, Madison, USA).

Quantitative RT-PCR was performed using a 7300 Real-Time PCR System® (Applied Biosystems, Warrington, UK) with SYBR-green.

Relative expression fold change for the respective genes was assessed using normalized cycle threshold values (ΔCT). Normalization was carried out against the expression in sort purified L chain<sup>+</sup>/CD80<sup>-</sup> cells of unstimulated splenic leukocytes.

## 2.9 ELISpot assays

On the day preceding the experimental procedure, nitrocellulose ELISpot microtiter plates (Merck KGaA, Darmstadt, Germany) were coated with 100 µl of anti-chL chain antibody solution (5 µg/ml in sterile PBS, pH 7.2 (Thermo Fisher Scientific, Waltham, USA)), sealed with an adhesive foil, and subsequently incubated overnight at 4°C. Following this incubation, any unbound antibody was removed by washing the plates with PBS. Subsequently, the wells were treated with a blocking solution consisting of PBS supplemented with 10% FBS and incubated for two hours at 40°C.

Blocking-buffer was discarded and the residual liquid was tapped out onto cellulose.

Cytokine-stimulated splenic leukocytes were isolated via density gradient centrifugation and adjusted to a density of 1x10<sup>5</sup> for IgM and 2x10<sup>5</sup> cells/ml for IgA and IgY analysis. Subsequently, 100 µl of this cell suspension was applied to each well of the plate. The plate was then incubated at 40°C with 5% CO<sub>2</sub> for a duration of 48 hours.

Following the incubation period, a triple washing step with PBS containing 0.01% Tween®-20 (ITW Reagents, Castellar del Vallès, Spain) was conducted to remove any nonspecifically bound material.

Biotin (Roche, Basel, Switzerland) was used to biotinylate Ig-specific antibodies (Table 2) and the antibodies were then added to the plate and allowed to incubate for one hour at room temperature in the dark. After another triple washing step with PBS-T, peroxidase-conjugated streptavidin was applied to the plate as the detection antibody.

After a further washing step with PBS, 50 µl of KPL TrueBlue™ Substrate (LGC Clinical Diagnostics, Teddington, UK) was added to each well and allowed to incubate for five minutes. The reaction was terminated upon the appearance of intense blue staining in the spots by discarding the remaining substrate and promptly washing the plate with distilled water.

Antibody secreting cells, visible as blue spots, were analyzed using the Bioreader® 7000 V machine (BIOSYS Scientific Devices GmbH, Karben, Germany) with the EazyReader software (Version 20.9, Patch 143) that allows for automated counting of the spot number as well as the analysis of total optical density (TOD = integral accumulation of area and intensity of each spot).

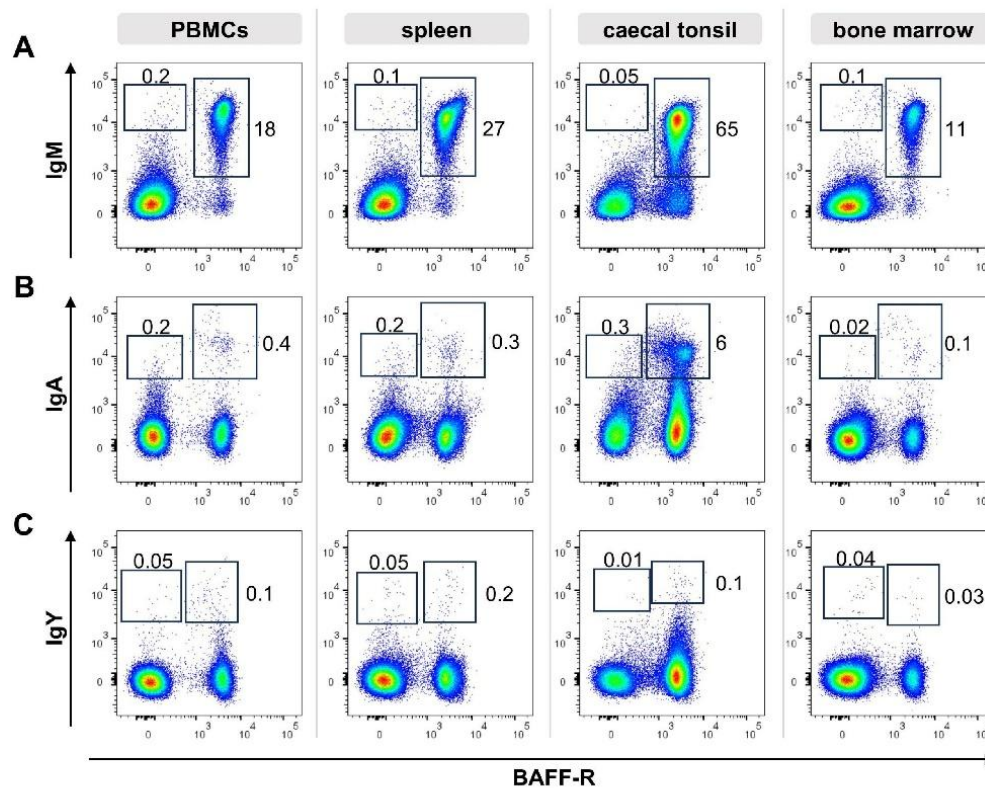


FIGURE 1

Ig surface expression in lymphoid tissues. Leukocytes were isolated from the respective tissues of a young adult chicken and analyzed by flow cytometry for the expression of surface IgM (A), IgA (B), IgY (C) and BAFF-R. Plots are gated for viable, single CD45<sup>+</sup> lymphocytes. Data are representative for four independent experiments.

## 2.10 IBDV ELISA

The presence of IgY antibodies specific to the IBDV VP2 protein was assessed in serum samples using a commercial indirect ELISA kit (ID Screen<sup>®</sup> IBD VP2, Innovative Diagnostics, France). Antibody titers were calculated according to the manufacturer's instructions. The IBD immune status is considered positive if the antibody titer is greater than or equal to the cut-off value of 1002.

## 2.11 Statistics

Data were analyzed using one-way or two-way ANOVA with subsequent Bonferroni multiple comparisons in GraphPad Prism 5.04 (GraphPad Software, Boston, USA).

## 3 Results

### 3.1 slg class-switched cells are hardly detectable in non-mucosal tissues

So far, no specific markers for chicken plasma and memory B cells were described. Since class switch of the B cell receptor (BCR)

can be one part of the B cell differentiation process and antibodies for all three chicken immunoglobulin (Ig) isotypes are available, we started our search for differentiated peripheral B cells by flow cytometric analysis of Ig expression on B cells from blood, spleen, caecal tonsil and bone marrow. Therefore, cells were stained for CD45 as a pan-leukocyte marker, the B cell marker BAFF-R and IgM, IgA or IgY and at least 100,000 CD45<sup>+</sup> viable single cells were counted (Figure 1).

In all tissues, the vast majority of B cells expressed surface IgM (sIgM), (96% of B cells in the blood, 98% in the spleen, 87% in the caecal tonsil and 98% in the bone marrow, Figure 1A). However, we consistently detected a small but distinct population of BAFF-R<sup>+</sup> B cells expressing surface IgA (sIgA) with 3%, 1.4% and 1.6% in blood, spleen and bone marrow. With 12.7%, B cells from the gut associated caecal tonsil contained a much higher amount of IgA expressing B cells (Figure 1B). Although, IgY is the dominant immunoglobulin in chicken serum (53–55), no distinct IgY<sup>+</sup> B cell population was detectable in the analyzed tissues. In spleen and bone marrow a few IgY<sup>high</sup> cells were found, but other positive cells presented largely as a IgY<sup>low</sup> smear above the isotype control background (Figure 1C). Mean frequencies of sIg<sup>+</sup>/marker<sup>+</sup> cells are shown in Supplementary Table 1.

Most of the surface Ig positive cells were also positive for the BAFF-receptor, defining them as B cells. However, to a variable



extent in all tissues and for all three immunoglobulins, additional Ig<sup>low</sup>/BAFF-R<sup>+</sup> cells were detected. In order to verify this observation, the staining was repeated with a second B cell marker chB6, which also demonstrated chB6<sup>+</sup> BCR<sup>+</sup> cells (Supplementary Figure 2). Hence, based on the expression of the B cell markers BAFF-R and chB6, two different Ig positive populations can be discriminated: i) (B cell) marker-positive, Ig positive cells and ii) marker-negative/Ig positive cells. As immunohistochemistry had shown chicken plasma cells as Bu1 negative cells or as cells that show only very weak staining (21), the later population could contain plasma cells.

It should be noted that BAFF-R and chB6 staining in the spleen and caecal tonsil were not entirely homogeneous. In many animals, a small, variably well-defined subpopulation with higher expression levels was observed (Supplementary Figure 2).

### 3.2 Establishment of a CD40L/IL-10 based *in vitro* culture system for B cell differentiation

As the frequency of class-switched cells among freshly isolated B cells was quite low and the origin of BAFF-R/Bu1/Ig<sup>+</sup> cells remained unclear, we aimed to differentiate chicken B cells *in vitro* to follow the differentiation process and get enough cells for phenotyping. Therefore, we combined CD40L, which was already shown to induce *in vitro* proliferation of chicken B cells (23) with chicken IL-10.

#### 3.2.1 CD40L and IL-10 act synergistically on short term proliferation

Analysis of [<sup>3</sup>H]-thymidine uptake in splenocyte cultures 48h post stimulation showed that IL-10 alone did not induce any cell proliferation. Stimulation with CD40L led to substantial proliferation but the combination of both CD40L and IL-10 resulted in a significant synergistic effect (Figure 2A). Titration for optimal concentration of IL-10 is depicted in Supplementary Figure 3.

The synergistic effect of both cytokines was also seen when cultures were analyzed by flow cytometry. Here, after 48h the fraction of blasting FSC<sup>high</sup> (proliferating) cells in CD40L and IL-10 stimulated cultures was almost 3fold higher than with CD40L alone (33% vs. 13%). In addition, staining for L chain demonstrated that after CD40L/IL-10 stimulation more than 90% of FSC<sup>high</sup> cells are L chain<sup>high</sup> B cells (Figure 2B, the corresponding CD3 staining is shown in Supplementary Figure 6).

#### 3.2.2 Early transcriptomic and phenotypic changes by CD40L and IL-10 stimulation

Flow cytometric analysis of CD40L/IL-10 stimulated splenocytes after 48h showed a decreased expression of the two B cell markers chB6 and BAFF-R within proliferating FSC<sup>high</sup> cells compared to the non-proliferating subset. We also found cells of medium FSC with high chB6 and low BAFF-R expression, which were most likely derived from the small chB6<sup>high</sup> subpopulation, which was present in these birds.

Strikingly, as demonstrated by the largely identical L chain staining between small and large cells, expression of the BCR was not affected and L chain positive cells represented the vast majority of proliferating blasts. In addition, CD80 was strongly upregulated on proliferating cells and this costimulatory molecule was exclusively found on FSC<sup>high</sup> cells (Figure 2B).

Based on this correlation of size increase and CD80 expression, we sort purified chB6<sup>+</sup>/CD80 positive (large) and chB6<sup>+</sup>/CD80 negative (small) B cells after 2 days and also after a longer culture of 4 days (Figures 2C–I) and analyzed the expression of selected markers for B cell differentiation and stage specific transcription factors by qRT-PCR in comparison to freshly isolated splenic B cells.

In general, strong differences were detected between small and blasting cells and also for both populations in comparison to fresh cells over time.

As shown for the protein by flow cytometry, *chB6* mRNA abundance was likewise reduced in activated CD80<sup>+</sup> cells compared to CD80<sup>−</sup> at day 2 and showed further strong reduction in CD80<sup>+</sup> blasts at day 4 (Figure 2B).

No significant effects of CD40L/IL10 stimulation were detected of the BCR component *CD79b*, when groups were compared over time. However, as the tendency to higher (CD80<sup>−</sup>) and lower (CD80<sup>+</sup>) expression was oppositional, differences on the  $\Delta$ CT level between small and blasting cells were significant. This picture does not exactly meet the increased L chain surface staining of proliferating cells, but confirms that stimulated cells do not loose BCR surface expression (Figure 2B).

$\mu$ S, the secretory form of IgM was increased in both groups at day 2 and in day 4 CD80<sup>−</sup> cells (7, 10 and 6fold) and showed a strong 100fold increase in CD80<sup>+</sup> cells at day 4 (Figure 2E).

Among the analyzed transcription factors, the expression of both the B cell-specific transcription factor *PAX5* and the germinal center/memory cell-associated transcription factor *BCL-6* were reduced in CD80<sup>+</sup> cells (Figures 2F, G), while they remained fairly constant (*Pax5*) or showed a strong upregulation (*BCL-6*) in the CD80<sup>−</sup> population. In contrast, *BLIMP1*, the plasma cell specific transcription factor, revealed the opposite profile and was strongly upregulated in CD80<sup>+</sup> cells (Figure 2H).

Regarding its essential role for class switch recombination, we also measured expression of *AICDA*. We found a significantly increased *AICDA* abundance in all groups compared to day 0, but between small and blasting cells no significant differences were detected (Figure 2I).

#### 3.2.3 Cell composition in extended cultures

Prompted by these results and the knowledge that in cultures stimulated solely with CD40L the plasmablast phenotype becomes more prominent after a longer period of cultivation (23), we extended the culture of CD40L/IL-10 stimulated cells up to six days.

To examine the cellular composition of these total spleen leukocyte cultures, cells were analyzed by flow cytometry every second day. While in the first two days the frequency of living cells dropped to around 45% (data not shown), the number of viable cells was only reduced to 75% of the starting material and about one



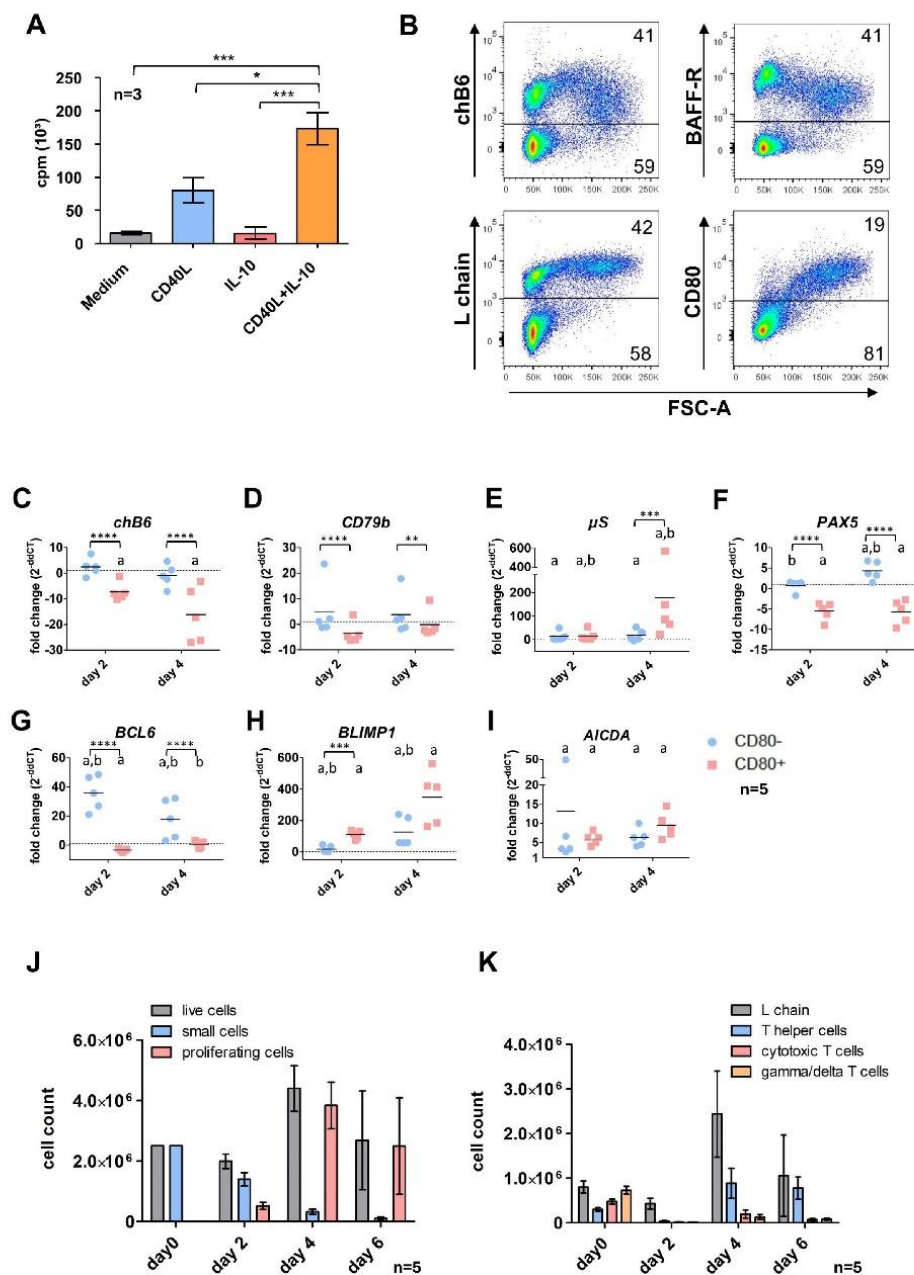


FIGURE 2

Effects of CD40L and IL-10 stimulation on spleen leukocytes. (A) Splenic leukocytes were isolated and incubated in the presence of the respective cytokines or medium only. After 48h [ $^3$ H]-Thymidine was added and cultures were harvested 16h later to assess proliferation. Shown are mean  $\pm$  SD, with\*:  $P \leq 0.05$ ; \*\*\*:  $P \leq 0.001$  by two-way ANOVA with Bonferroni *post hoc* test. Shown are mean  $\pm$  SD of three independent experiments. (B) Cells were stimulated for 48h with CD40L/IL-10 and analyzed by flow cytometry. Plots are gated for viable, single leukocytes. (C–I) L chain $^+$ /CD80 $^+$  and L chain $^+$ /CD80 $^-$  cells were sort purified from CD40L/IL-10 stimulated cultures after 2 and 4 days. Expression of the respective genes was determined by qRT-PCR and normalized to freshly isolated, sort-purified L chain $^+$ /CD80 $^-$  cells by the  $2^{\Delta\Delta CT}$  method. Shown are individual values and mean of five animals. Statistics were calculated by two-way ANOVA with Bonferroni *post hoc* test on  $\Delta CT$  values. Asterisks indicate statistical differences between the groups at a single time point, while letters indicate statistical differences within the population between the different time points. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; \*\*\*\*:  $p \leq 0.0001$ ; a:  $p \leq 0.05$  compared to d0; b:  $p \leq 0.05$  comparison between day2/day4. (J, K) Splenic leukocytes were stimulated with CD40L/IL-10 for 6 days. At the indicated timepoints, cells were analyzed by flow cytometry populations according to size (J, gated for viable, single leukocytes) or surface marker expression (K, gated from viable, single leukocytes (day 0) and viable, single proliferating leukocytes (d2–6)). Helper T cells were addressed as CD4 $^+$  cells, cytotoxic T cells as CD8 $^+$ /TCR1 $^+$  and  $\gamma\delta$  T cells as TCR1 $^+$ . Shown are mean  $\pm$  SD of five animals.

quarter of the cells presented as proliferating blasts. While small cells were continuously lost from culture, the number of blasting was strongly increased at day 4 and day 6 (representing 90% of all cells), and consequently also the total number of living cells (Figure 2J).

As we started with a mixed culture of splenic B and T cells, we examined the cultures for the presence of different lymphocyte populations among the blasting cells. As shown in Figure 2K after 2 days, the blast population contained around 90% B cells and only few proliferating helper, cytotoxic and  $\gamma\delta$  T cells were found. From day 2 to day 4 of culture B cell blasts increased 6fold in number (from  $4 \times 10^5$  to  $2.4 \times 10^6$ ) indicating strong proliferation among these cells.

Strikingly, T helper cell numbers show a 25fold increase between day 2 and day 4 and hence, these cells exhibit the highest proliferation rate at this timepoint and at day 6, cultures contained roughly the same amount of L chain and CD4<sup>+</sup> cells. In addition, we also found that cytotoxic and  $\gamma\delta$  T cells start to proliferate and between day 2 and day 4 their numbers increased 14 and 10-fold, respectively.

Except for certain T helper cell cultures, most cell populations exhibited a decrease in cell numbers by day 6. In the case of B cell blasts, this decline was observed across all cultures, although the extent varied significantly, ranging from 70% to 16% of the cell count on day 4. This reduction may be partially attributed to suboptimal growth conditions following the proliferation peak on day 4.

Hence, extended culture periods after CD40L/IL-10 stimulation led to an initially strong growth of B and to later timepoints also induced T cell proliferation.

### 3.2.4 CD40L/IL-10 induces both increased Ig production and class switching

To analyze Ig production in CD40L/IL-10 stimulated cultures, splenocytes were stimulated for 6 days with CD40L or CD40L/IL-10 and antibody production was measured via ELISpot assay (Figure 3A).

Hardly any Ig producing cells were detectable in media controls, but the addition of CD40L resulted in clearly visible spots for IgM and IgY while only very few IgA<sup>+</sup> cells were found. The combination of CD40L and IL-10 had a strong synergistic effect on the secretion of all three Ig isotypes and led to a 4fold higher number of IgM<sup>+</sup> spots and a 5fold increase of IgY<sup>+</sup> spots and a 7fold increase of IgA<sup>+</sup> spots (Figure 3B). The strong synergism of CD40L/IL-10 was also observed for the total optical density (TOD), the integral accumulation of area and intensity of each spot, which next to the number of antibody secreting cells also includes the secreted amount of antibodies (Figure 3C).

As the ELISpots had revealed the presence of class-switched, antibody secreting cells in the CD40L/IL-10 stimulated cultures, we examined whether this was also linked to changes in BCR surface expression. When cells were analyzed for Ig surface staining by flow cytometry at different timepoints, IgM remained the dominant surface immunoglobulin over the stimulation period of 6 days (93, 75, 73% of the B cells at days 2, 4 and 6, respectively). But in addition to IgM, a small but clearly defined population of IgA<sup>+</sup> cells was detectable after 4 and 6 days representing 4% of L chain positive cells. And the culture system worked even better for class switch to

IgY, as after 4 days about one quarter of the B cells in culture expressed an IgY type BCR (Figure 3D). Importantly, class switch occurred only in the blasting cells of the cultures, the remaining small B cells maintained a BCR of IgM isotype (Figures 3E, F).

## 3.3 Phenotype of *in vitro* generated plasma blasts

Having established a culture system that induces B cell proliferation, class switching, and antibody secretion, we next conducted flow cytometric phenotyping of the differentiating B cells. Analysis after 2 days had already shown that L chain expression remained high after CD40L/IL-10 stimulation. More prolonged examination revealed a constantly increased amount of surface BCR after 4 and 6 days, though the MFI for L chain staining on day 6 was slightly lower (Figures 4A, J). Thus, L chain staining picks up all B cells in stimulated cultures and for further phenotyping of B cell blasts, all samples were gated for large L chain positive cells.

The initially observed downregulation of BAFF-R expression continued stepwise after 4 and 6 days, resulting in an almost complete loss of this cytokine receptor at day 6 (Figures 4B, K). We also detected a further loss of chB6 within the proliferating cells, but in contrast to BAFF-R after 2 or 4 days two subpopulations became detectable, one with reduced chB6 expression and a smaller one which became nearly chB6 negative (Figures 4C, L). Hence, only a subpopulation of the cells can be considered as marker-negative/Ig positive cells. The variability between individual animals in the timing of the appearance of the two populations and their size resulted in a lack of statistical significance (Figure 4L).

The activation markers CD80 and CD25 showed a similar staining pattern, with a strong upregulation after 2 days followed by a strong drop at day 4 and 6. However, they differ in their initial expression as freshly isolated B cells are all CD80 negative while approximately one quarter of the cells show a medium CD25 expression. While CD25 expression returned to baseline levels at 0 hours, CD80 expression remained slightly and significantly elevated (Figures 4D, E, M, N).

The pattern of increased marker expression after two days followed by a downregulation at later timepoints was in general also observed for the chemokine receptors CXCR4 and CXCR5. They differ only in that CXCR4 was hardly found on freshly isolated cells, whereas all B cells from the spleen were CXCR5 positive (Figures 4F, G, O, P).

MHC class II expression, as indicator of the antigen presenting capacity of the cells showed an about 5fold increased expression at day 2 (Figures 4H, Q) and stayed significantly increased over time. Interestingly, in most animals we found a transient reduction in MHCII expression on day 4 followed by a renewed increase on day 6.

CD57 has been proposed as a possible plasma cell marker in chickens (56). Only a very small fraction of freshly isolated splenic B cells expresses CD57, but CD40L/IL-10 stimulation induced a 60fold upregulation of CD57 on day 2, and only a small proportion of cells remains negative. This pattern remained largely unchanged at day four and six of stimulation, though in parallel to MHCII, on day 4 a transient reduction of CD57 from a



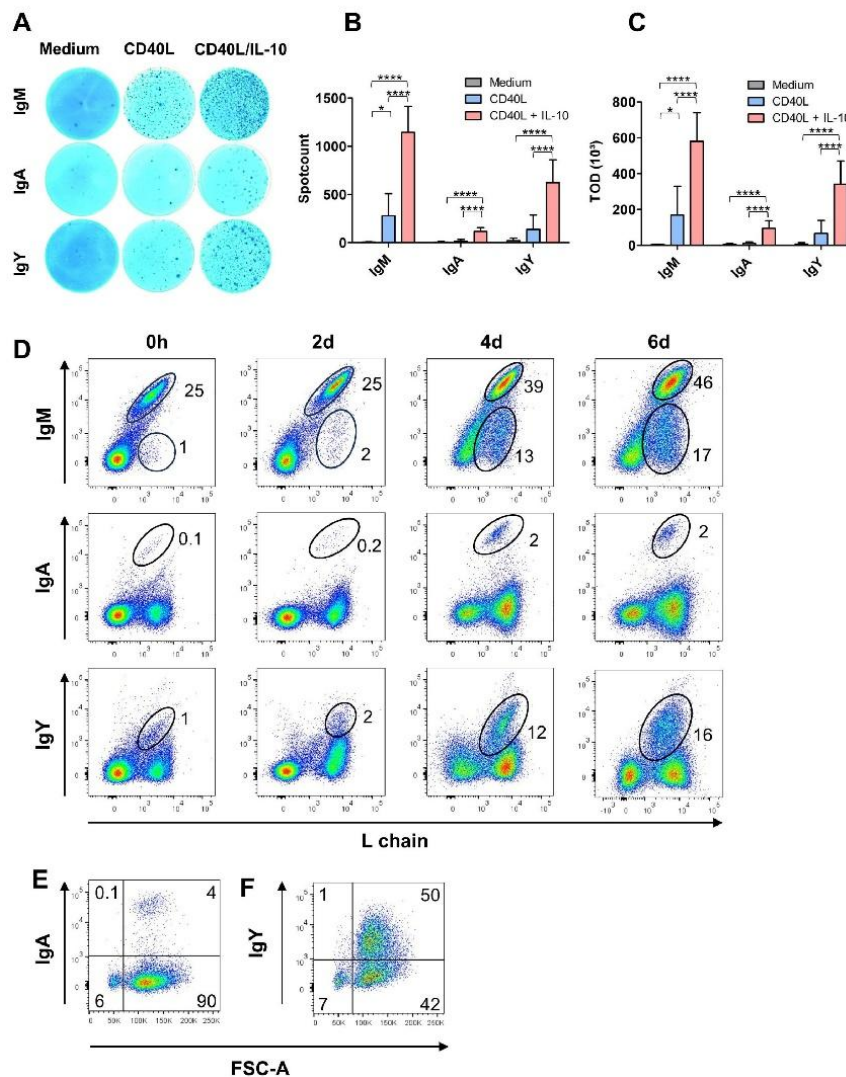


FIGURE 3

CD40L/IL-10 stimulation induces Ig secretion and class switch. (A–C) ELISpot-assays for total IgM, IgA and IgY from splenic leukocytes after six days of culture in the presence of the respective cytokines. (A) Representative photos from an ELISpot plate, (B) spot count (B) and (C) total optical density (TOD): Shown are mean  $\pm$  SD of three independent experiments with \*:  $P < 0.5$ ; \*\*\*\*:  $P < 0.0001$  by one-way ANOVA with Bonferroni *post hoc* test. (D) Surface expression for IgM, IgA and IgY was determined by flow cytometry on freshly isolated and CD40L/IL-10 stimulated cells after the indicated times of culture. Blots are gated for viable, single leukocytes. (E, F) Size (FSC) of class-switched cells after six days of CD40L/IL-10 stimulation. Blots are gated on L chain<sup>+</sup>, viable, single leukocytes. (D–F) Blots are representative for three independent experiments.

median MFI of about 5000 below 4000 could be observed (Figures 4I, R). Gating for small and blasting Ig<sup>+</sup> cells revealed that CD57 upregulation is largely restricted to blasting cells (Figure 5A). Relating CD57 and Ig surface expression, showed that IgA<sup>+</sup> and IgY<sup>+</sup> class-switched cells can be found both in the CD57<sup>+</sup> and CD57<sup>-</sup> fraction (Figure 5B). Hence, CD57 expression accompanies blast formation but CD57 expression and class switch seem to occur independently.

In summary, CD40L/IL-10 stimulated B cells retain BCR positivity, exhibit a continuous loss of BAFF-R and chB6 expression, display a moderate upregulation of MHC class II, and show a marked upregulation of CD57. For the activation markers and chemokine receptors analyzed, an initial activation peak at day 2 is followed by a

subsequent downregulation of these markers, clearly delineating an early activation phase from later time points in the culture.

### 3.4 Transient expression of TACI and CD138 during *in vitro* B cell differentiation

Though phenotyping of CD40L/IL-10 differentiated B cells has revealed interesting staining patterns, no specific marker for those cells was identified among the available mAbs. As human plasma cells can be addressed by CD138 expression (13, 14) and both memory B and plasma cells show a switch from BAFF-R to TACI expression, we generated new chicken specific antibodies

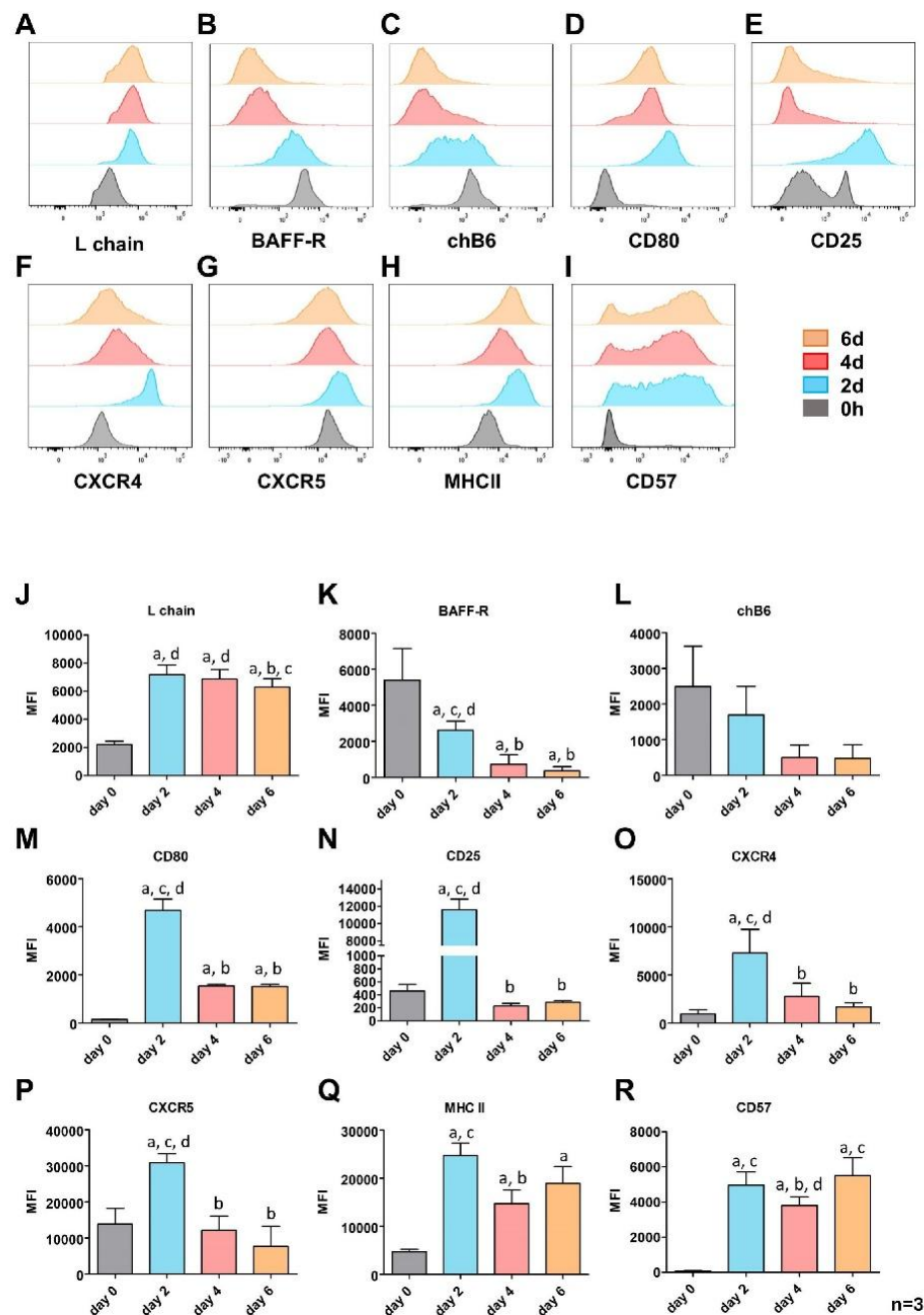


FIGURE 4

CD40L/IL-10 induced expression of surface markers in prolonged cultures. Splenic leukocytes were stimulated with CD40L/IL-10 for six days and analyzed for the expression of the indicated surface markers by flow cytometry analysis every second day. Populations are gated for L chain<sup>+</sup>, viable, single leukocytes at day 0 and proliferating (large), L chain<sup>+</sup>, viable, single leukocytes for the subsequent time points. (A–I) Surface marker expressions for one representative out of three experiments. (J–R) Mean  $\pm$  SD of the mean fluorescence intensity (MFI; median) for three independent experiments. Statistics were calculated by two-way ANOVA with Bonferroni *post hoc* test. Letters indicate significant differences with  $p \leq 0.05$  to day 0 (a), 2 (b), 4 (c) or 6 (d) of culture.

for these molecules (Staining on transfected cells is shown in [Supplementary Figure 4](#)).

Staining of CD40L/IL-10 stimulated cells revealed that freshly isolated cells are vastly negative for both markers. However, after two days in the presence of CD40L/IL-10 two thirds of the blasting

B cells expressed CD138 and one third expressed TACI ([Figures 6A, B](#)). However, distinct expression of both markers declined at later timepoints.

Analysis of light chain positive, proliferating cells revealed that at day two, nearly all cells that were positive for chTACI also



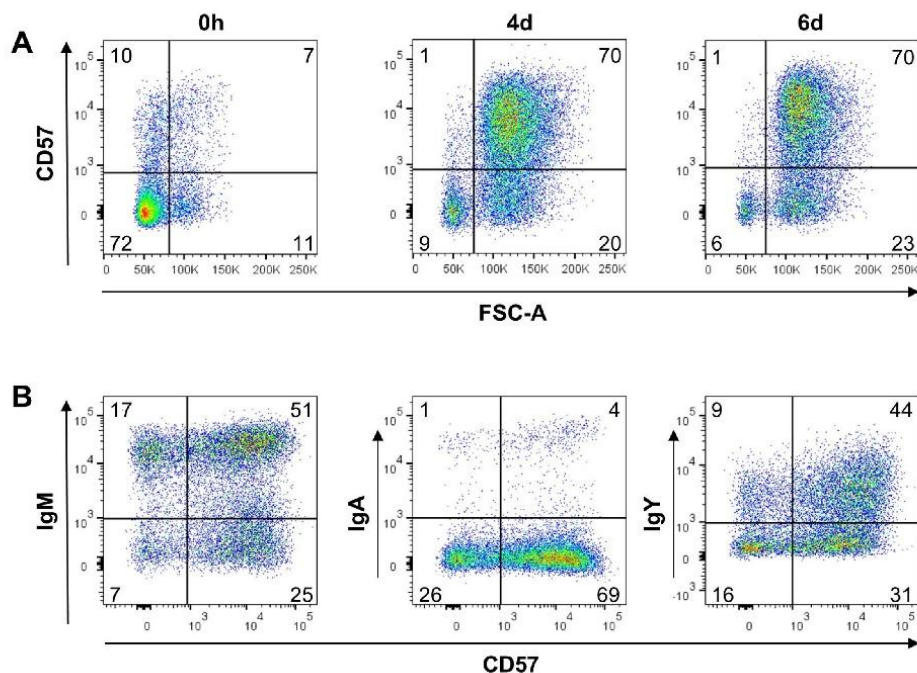


FIGURE 5

Increased CD57 expression is restricted to blasting cells in stimulated splenocytes. Splenocytes were stimulated with CD40L/IL-10 and analyzed for CD57 expression by flow cytometry. Shown data are representative for three independent experiments. (A) Size (FSC) of CD57 expressing L chain<sup>+</sup> cells at the indicated timepoints; blots are gated for L chain<sup>+</sup>, viable, single leukocytes. (B) CD57 expression on class-switched cells after six days of culture; blots are gated on blasting, L chain<sup>+</sup>, viable, single leukocytes.

expressed chCD138 but the majority of chCD138 positive cells remained negative for chTACI (Supplementary Figure 7A). As BAFF-R and TACI are both receptors for the cytokine BAFF, we examined the co-expression of TACI and BAFF-R, on light chain positive proliferating cells. After two days, all TACI-expressing cells were also BAFF-R<sup>+</sup>. But at later timepoints, when expression of both receptors decreased, a small proportion of the cells still expressed a low amount of BAFF-R, but the majority of light chain positive cells expressing neither of both BAFF receptors (Supplementary Figure 7B).

Among freshly isolated leukocytes from different lymphoid tissues below one percent of the cells was positive for CD138 and TACI. The majority of positive cells were L chain negative/non-B cells. The only exception were caecal tonsil leukocytes, where the few CD138<sup>+</sup> and TACI<sup>+</sup> cells were L chain<sup>+</sup> (Figures 6C).

Hence, neither CD138 nor TACI seem to be expressed throughout the differentiation process to plasma cells but rather at a short time window after initial cell activation.

### 3.5 Distribution of CD57 expression on primary B cells

Since the *in vitro* system had revealed an interesting staining pattern for CD57 on proliferating and class-switched cells, we investigated CD57 expression on primary B cells from different lymphoid tissues. Only a fraction of B cells from all analyzed organs

was CD57<sup>+</sup>. The lowest proportion of CD57<sup>+</sup> cells was found among unswitched IgM<sup>+</sup> cells ranging from 4% in spleen up to 7% in the blood. A much higher percentage of CD57<sup>+</sup> cells was detected in cells post class switch to IgA with about 30% of IgA<sup>+</sup> cells in blood, CTs and bone marrow expressing CD57 while around 20% of IgA<sup>+</sup> blood B cells expressed CD57. Due to the detection of only a few IgY<sup>+</sup> cells, clear evidence is lacking; however, it appears that the majority of IgY<sup>+</sup> cells do not express CD57 (Figures 7A–C).

Relating CD57 expression on L chain positive cells to chB6 downregulation demonstrated that in all organs much more marker-negative cells expressed CD57 (40–67%) compared to the marker-positive fraction (3–5%) (Figures 7D–F). This could be indicative of a higher activation level of these cells, but our data do not show that CD57 expression is indicative of a defined B cell differentiation stage as memory or plasma cells.

### 3.6 Influence of age and vaccination

To analyze the influence of bursa involution and frequent immune responses over time, we determined the frequency of class-switched cells in blood and spleen of commercial layers at the age of seven weeks (fully developed bursa), 17 weeks (beginning bursa involution, before the start of laying), 27 weeks (no bursa, laying) and 70 weeks (end of laying, before moult).

Overall, in these birds, percentages of both chB6<sup>+</sup> and chB6<sup>-</sup> BCR positive cells in the blood were quite low, but we detected more

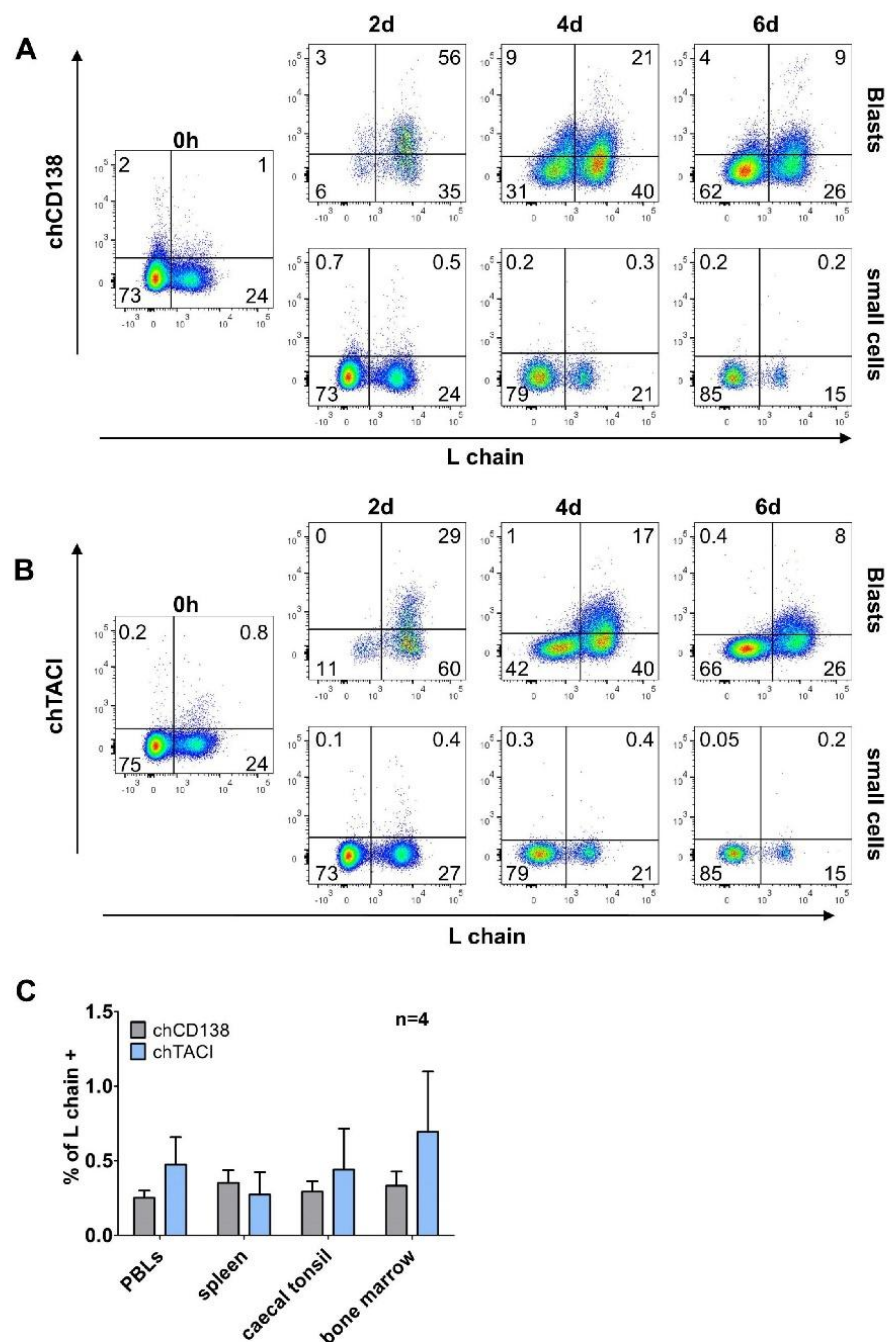


FIGURE 6

Time-limited expression of chCD138 and chTACI on activated B cells in splenocyte cultures. (A, B) Splenocytes were stimulated with CD40L/IL-10 and analyzed by flow cytometry for expression of chTACI (A) or chCD138 (B) at the indicated timepoints. Blots are gated for viable, single leukocytes and either large, blasting cells or small cells are shown. Data are representative of three independent experiments. (C) Expression of CD138 and TACI on L chain<sup>+</sup> cells from different lymphoid organs. Flow cytometry blots were gated for viable, single CD45<sup>+</sup>/L chain<sup>+</sup> leukocytes. Shown are mean  $\pm$  SD of four independent experiments.

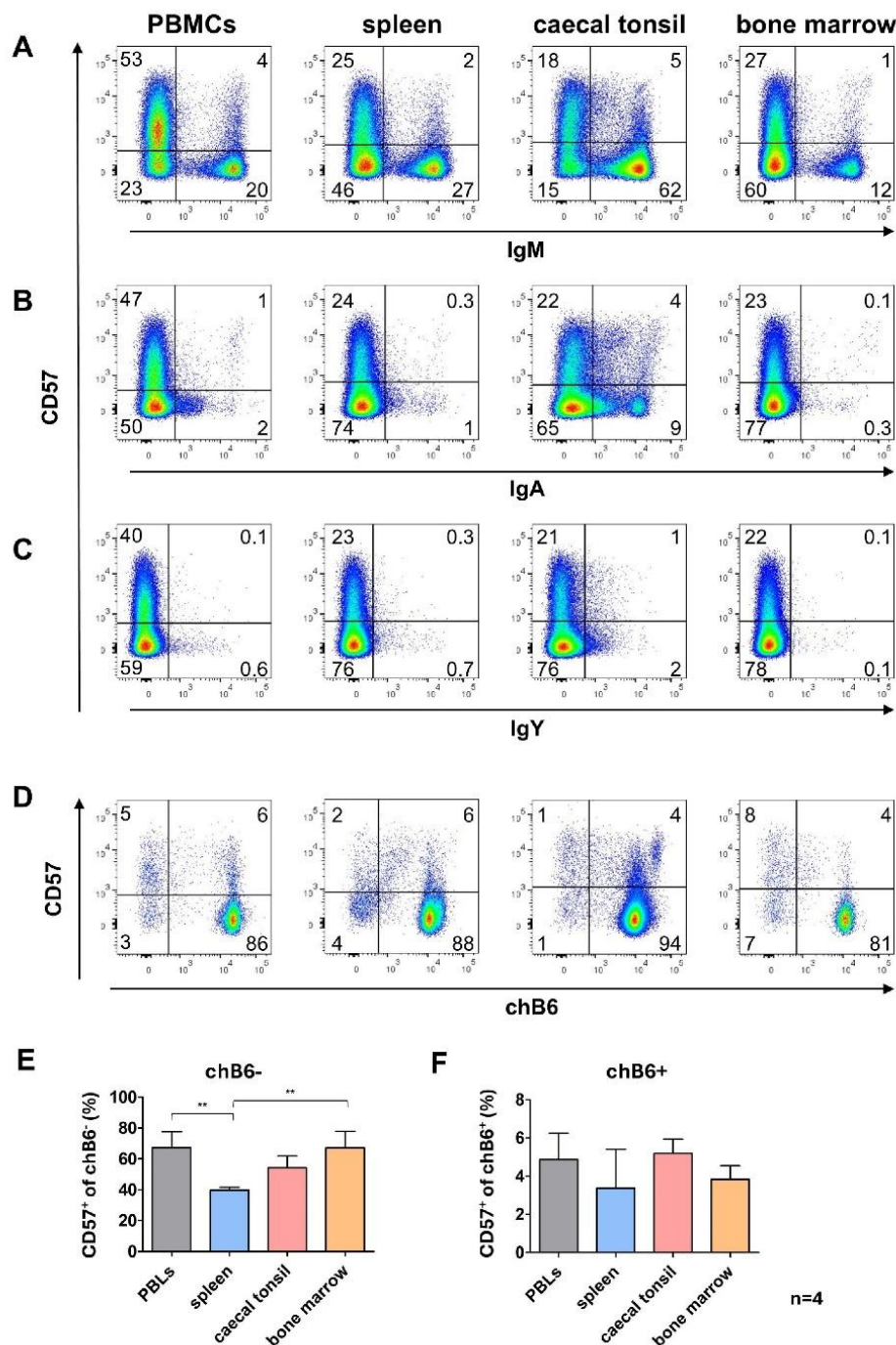


FIGURE 7

CD57 expression in lymphoid tissues. Leukocytes were isolated from the indicated tissues and analyzed for CD57 expression by flow cytometry. (A–C) CD57 expression on IgM<sup>+</sup> and Ig class-switched cells, with blots gated for viable, single leukocytes. (D) CD57 expression on marker-negative (chB6<sup>-</sup>) and marker-positive (chB6<sup>+</sup>) L chain positive leukocytes. Blots are gated for viable, single, CD45<sup>+</sup> cells. Shown are representative blots of four independent experiments. (E, F) Proportion of CD57<sup>+</sup> cells among chB6<sup>-</sup>/L chain<sup>+</sup> (E) and chB6<sup>+</sup>/L chain<sup>+</sup> cells (F). Statistics were calculated by one-way ANOVA with Bonferroni *post hoc* test with \*\*:  $p \leq 0.01$ ,  $n=4$ .



chB6<sup>+</sup> IgA and IgY positive cells (the potentially class-switched plasma cells) than unswitched chB6<sup>+</sup>/IgM<sup>+</sup> cells. Highest percentages for both chB6<sup>+</sup> and chB6<sup>+</sup> IgM<sup>+</sup> and IgA<sup>+</sup> cells and chB6<sup>+</sup>/IgY<sup>+</sup> cells were detected at 17 weeks just before laying. Only chB6<sup>+</sup>/IgY<sup>+</sup> cells peaked later at 27 weeks (Figure 8A). The splenic B cell compartment was characterized by a strong and continuous increase in chB6<sup>+</sup>/IgM<sup>+</sup> cells over time. For all other populations only very low and quite constant frequencies were detected (Figure 8B). Hence, we could not detect a distinct increase of class-switched cells over time, only the begin of laying seems to have some effect.

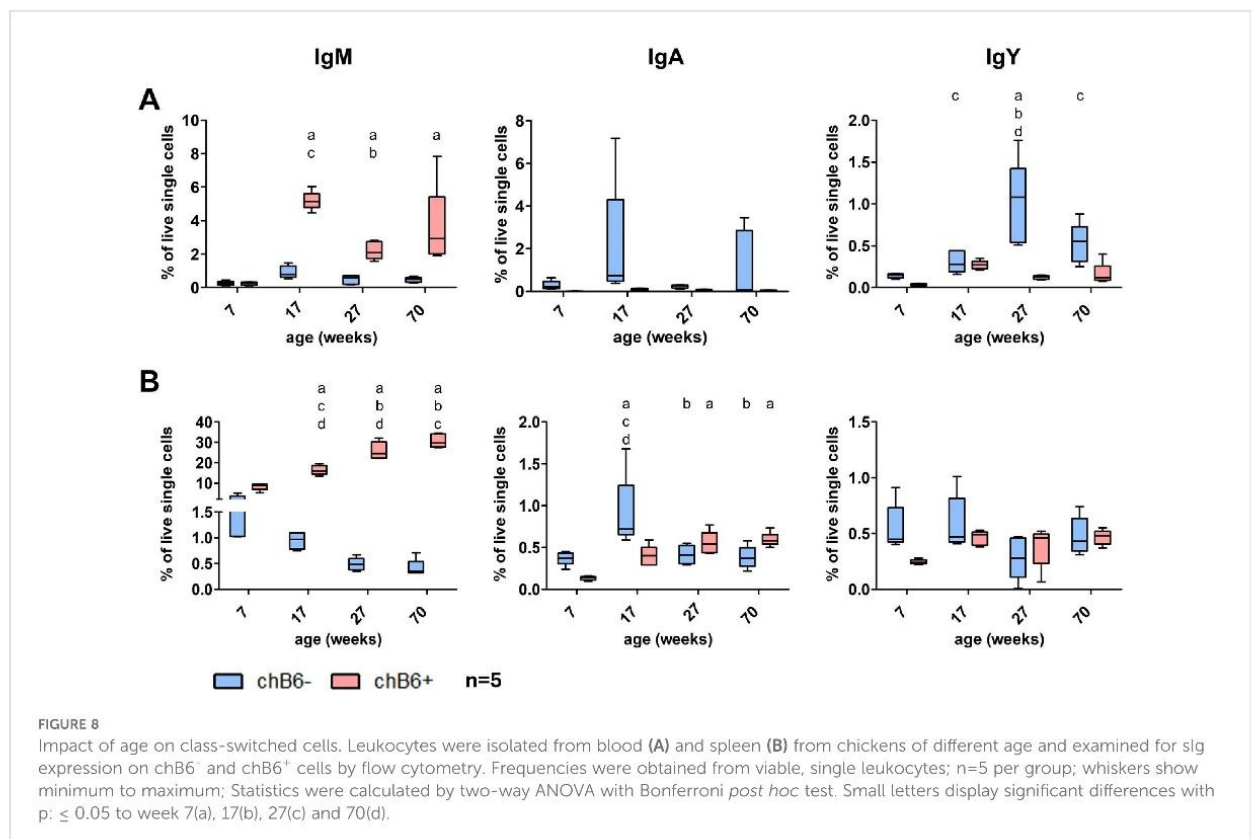
Finally, we assessed whether we could detect an influence of vaccination on the presence of class-switched cells. Therefore, chickens were vaccinated with an intermediate virulent IBDV vaccine strain. This vaccine is reported to induce acute B cell death in the bursa in the first week p.v. but also high IBDV specific antibody titers (57). We found that at day 28 p.v. the peripheral B cell system is recovering, though the fraction of peripheral B cells is still reduced (Figure 9A). We measured homogeneously high antibody titers in all birds and as the ELISA system detects IgY we could also demonstrate class switch (Figure 9B). When we analyzed Ig expressing cells in the blood, we detected a significantly lower percentage of IgM<sup>+</sup>/BAFF-R<sup>+</sup> cells in vaccinated birds, but all other chB6<sup>+</sup> and chB6<sup>+</sup> Ig<sup>+</sup> populations showed a highly significant increase in vaccinated birds (Figures 9C, D). Thus, IBDV vaccination has led to a substantial increase in chB6<sup>+</sup> cells and a significantly increased fraction of class-switched cells.

## 4 Discussion

In chickens little is known about class switch, affinity maturation, live time and location of plasma and memory cells, but a better understanding of these processes is necessary to develop more precise and specific vaccination strategies.

Due to the absence of specific differentiation markers for chicken B cells like CD19, CD20, CD27, CD38 and CD138, our approach was to discriminate naïve from differentiated B cells by identifying cells that have undergone Ig class switching. Overall, we found relatively few IgY and IgA positive cells in the analyzed tissues with frequencies from 1% to 2% of B cells in blood, spleen and BM. For humans it was shown that about 20 to 25% of blood B cells are class-switched cells (38, 58, 59), the majority of them representing memory cells (60). Hence, the frequencies observed for the chicken were substantially lower.

Only in the caecal tonsils, which are gut-associated secondary lymphoid structures rich in GCs, we identified a significant population of IgA<sup>+</sup> class-switched cells (7). This had already been shown by immunohistochemistry (61, 62), now we demonstrate that it is also possible to detect class switch to IgA by surface staining for flow cytometry. We could not detect a significant number of IgY-positive cells in any of the tissues examined, including the bone marrow, where in mammals long lived IgG<sup>+</sup> plasma cells (63–65) reside in specific survival niches. This is in accordance with observations from De Geus and colleagues, who by ELISpot also detected no differences in the number of IgY secreting





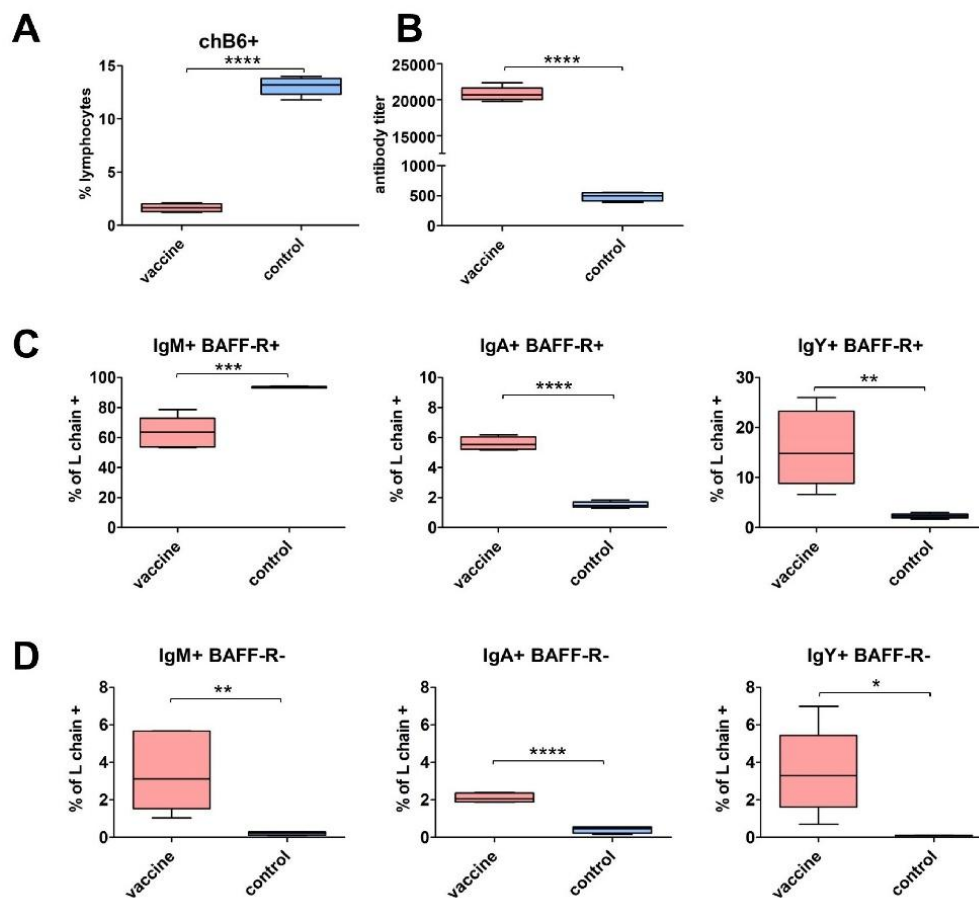


FIGURE 9

Impact of IBDV vaccination on class-switched cells. Six week old birds were vaccinated with a IBDV live vaccine and blood samples were analyzed 28 days post vaccination in comparison to unvaccinated controls. (A) Frequency of B cells (chB6<sup>+</sup>); (B) IBDV specific IgY antibody titer in sera were quantified by ELISA. (C, D) Percentage of Ig surface expressing cells from marker-positive (L chain<sup>+</sup>/BAFF-R<sup>+</sup>) (C) and marker-negative (L chain<sup>+</sup>/BAFF-R<sup>-</sup>) (D) B cells. n=5 in vaccinated group and n=3 in control group; whiskers show minimum to maximum. Frequencies were obtained from viable, single CD45<sup>+</sup> lymphocytes (A) and L chain<sup>+</sup> viable, single CD45<sup>+</sup> lymphocytes (C, D). (A–D) \*: p ≤ 0.05; \*\*: p ≤ 0.01; \*\*\*: p ≤ 0.001; \*\*\*\*: p ≤ 0.0001 by unpaired t-test.

cells between blood, spleen and bone marrow (66). However, given the very low frequency of plasma cells in human bone marrow preparations, which is reported to be below 0.5% (67, 68) our observations do not exclude the possibility of similar survival niches for IgY plasma cells in the chicken bone marrow.

#### 4.1 B cell differentiation *in vitro*

To enable the detailed characterization of a larger number of cells, we aimed to establish a culture system for chicken B cells that supports B cell differentiation and immunoglobulin class switching. We had already shown that trimerized soluble CD40L induces strong proliferation and long-term survival of chicken B cells (23). Based on reports that the addition of IL-10 to CD40 activated human B cells induced antibody secretion and class switch to IgG (50, 69) and the description of a functional chicken IL-10-homologue (51), we combined CD40L and IL-10 in cultures of mixed chicken spleen

leukocytes. We found a strong synergistic effect of CD40L and IL-10 on cell proliferation, which in the first days of culture was almost completely composed of B cells. Transcription factor analysis after two and four days of culture revealed very different profiles in small and blasting cells and demonstrated differentiation of the blasting cells toward plasma cells with a strong decrease in *Pax5* and increase in *Blimp1* expression (70). This was also supported by the upregulation of  $\mu$ S (the soluble form of IgM) (71). These ASCs were indeed detected in ELISpot assays with highly significant increases of spot numbers of all Ig isotypes. Compared to the Ig isotype distribution in freshly isolated cells, CD40L/IL-10 stimulation clearly changed the ratio toward isotype-switched cells with almost as many IgY and IgA ASCs than IgM secreting cells. In addition, we found strongly increased numbers of class-switched cells to both IgA and IgY by flow cytometric staining. This increase could be caused either by increased survival of the few class-switched cells in the starting population or the induction of class switch recombination (CSR). Supportive of the latter is the found upregulation of *AICDA*, the essential enzyme for CSR (72, 73), that we

observed in both small cells and blasts, and which was also reported for equally activated human B cells (50).

Thus, by stimulating splenic leukocytes with CD40L and IL-10, we established culture conditions leading to B cell proliferation, antibody secretion and an increased number of isotype-switched, preferentially IgY<sup>+</sup>, cells, which provide enough cells for a more detailed phenotypic analysis.

## 4.2 Phenotype of activated B cells

Surface staining for L chain expression on CD40L/IL-10 activated B cell blasts showed that development of ASCs did not induce BCR downregulation for any Ig isotype. This contrasts the long-time dogma for mammalian plasma cell differentiation, that cells switch from membrane-bound to secreted Ig expression (74–76), resulting in the loss of surface BCR expression. However, more recent data show an isotype dependent BCR loss. While IgG expressing plasma cells lose membrane bound Ig during their differentiation toward long lived plasma cells (77), IgM and IgA expressing plasma cells keep a functional BCR on their surface (77, 78). *In vitro* generation of plasma cells from human memory cells resulted in a similar picture with a reduction of BCR expression for IgM and IgA and a complete switch to intracellular Ig for IgG expressing cells (79). Our cultures were not extended beyond 6 days and culture conditions were not optimized for a terminal differentiation, which would require the withdrawal of CD40L (46, 79). Therefore, we cannot rule out that longer time of culture and different conditions could lead to BCR downregulation on chicken plasma cells. However, at least for this first phase of activation and ASC induction membrane expression of all BCR isotypes remained high, suggesting a different regulatory mechanism for IgY compared to IgG expression.

Beside the possible functional implications of this observation, like the continuous ability of these cells for antigen recognition, prolonged BCR expression was also of technical importance. In the absence of specific markers, it allowed us to gate the mixed leukocyte cultures for L chain<sup>+</sup> cells for a detailed phenotyping of differentiating B cells.

Like the BCR, MHC class II expression increased rapidly and remained at a very high level, demonstrating that B cells generated in our system keep both their capability to recognize antigen by the BCR and present antigen to T helper cells by MHC class II. Human B cells retain MHC class II expression in the GC and on memory and short-lived plasma cells and lose it only when differentiating into long-lived plasma cells (80), suggesting that this final differentiation step is not induced in our present system.

We found a reduced abundance of chB6 mRNA and a continuous reduction of surface expression from cell isolation to day six of CD40L/IL-10 stimulated cultures. This is in line with downregulation of chB6 on CD40L induced plasmablasts (23) and lack of chB6 staining on plasma cells detected by less-sensitive immunohistochemistry (21) and collectively suggests that chB6 expression is lost during the final differentiation of chicken B cells.

Interestingly, after two to four days of CD40L/IL-10 activation, chB6 expression became biphasic, with only part of the L chain<sup>+</sup> blasts showing a complete loss. This can either argue for an ongoing process toward a complete loss or indicates differentiation of different subpopulations and may represent a plasma and memory cell pathway, though *BCL-6*, the memory cell associated transcription factor was strongly upregulated in small cells.

BAFF-R, exclusively expressed on B cells and one of the chicken's two receptors for the B cell survival cytokine BAFF (25) was gradually and almost completely lost over the time of culture. In human B cell differentiation, only the plasma cell pathway (plasmablasts, short- and long-lived plasma cells) loses BAFF-R (81), while memory cells remain BAFF-R positive (82). This would argue against a possible memory B cell development in CD40L/IL-10 stimulated cultures. However, early prebursal and bursal chicken B cells (26) express BAFF-R, which is in contrast to BAFF-R expression only from the transitional B cell stage on in mammalian B cells (83, 84). This demonstrates significant differences in the expression profile of BAFF receptors between avian and mammalian B cells, which could also account for memory B cells.

Both, chB6 and BAFF-R are B cell markers which are expressed throughout chicken B cell development from the prebursal B cell stage onwards (85, 86). Their loss on differentiating culture cells along with consistent Ig surface expression makes it very likely that the observed population of BAFF-R<sup>+</sup>/chB6<sup>+</sup> (marker-negative) and Ig<sup>+</sup> cells, which we found *ex vivo*, represents further differentiated B cell stages and could contain plasma cells. But while in cultures a gradual loss of chB6 and BAFF-R was visible, cells in blood and tissues cells were mostly either marker-positive or marker-negative, with only few Ig<sup>+</sup> cells in spleen and CT showing an intermediate chB6 expression.

About one third of freshly isolated spleen B cells expressed CD25, the high-affinity chain of the IL-2 receptor. Among human blood B cells, the majority of memory B cells expresses CD25, while only 10 to 20% of naïve B cells and ASCs are CD25<sup>+</sup> (87). Another report describes the number of CD25<sup>+</sup> B cells in the blood with 2% and 7% in the spleen (88). Hence, the detected number of CD25<sup>+</sup> B cells in chickens was substantially higher and strongly enhanced by CD40L/IL-10 activation in the first days of culture. Interestingly, it vanished almost completely at later timepoints, highlighting that i) despite the continuous presence of both CD40L and IL-10 two distinct phases of activation and differentiation exist in these cultures and ii) either no memory cells are induced in these cultures or chicken memory cells do not express CD25.

CD80 expression also peaked on day two but lower levels of CD80 expression persisted at later time points. For human and murine B cells a strong upregulation of CD80 expression is associated with GC B cells (89, 90), it is partially retained on memory cells but lost during the differentiation of plasma cells (91). On the assumption that expression during chicken B cell differentiation is regulated similarly, that would imply an ongoing GC like differentiation process in our culture system.

The observed initial CXCR4 upregulation with a subsequent rapid loss of its expression is also in favor of a GC-like activity in



our culture system. Proliferating mammalian GC B cells upregulate CXCR4 to retain them in the CXCL12 high dark zone of the GC (92–94). Upon their final differentiation, memory and plasma cells then downregulate CXCR4, to allow them to leave the GC.

### 4.3 Applicability of CD57, CD138 and TACI

In mammals, CD57 is used as marker for cells of neural crest origin (95–97), and different NK (98, 99) and T cell subpopulations (100) (101). CD57 is a terminally sulfated carbohydrate epitope (glucuronic acid 3-sulfate) (102–104), but to date little is known on which molecules the CD57 epitope is actually expressed. Our analysis of chicken leukocytes from different tissues showed that the majority of non-B cells are CD57<sup>+</sup>. Since CD57 was reported as activation marker for chicken T cells (105), this suggests a similar expression pattern for mammalian and chicken T cells. In contrast, CD57 expression on human B cells is a rather rare event and only few B cells in the blood are CD57<sup>+</sup> (106), though a higher CD57 expression on B cells has been shown in patients with relapsing-remitting Multiple Sclerosis (106).

We investigated CD57 expression, as it was described as marker of chicken B cell activation and plasma cells (56) and could indeed detect a continuous upregulation of CD57 on most blasting cells post CD40L/IL-10 activation. Interestingly, throughout the whole culture period, a quarter of B cell blasts remained negative for CD57 and its expression was independent of Ig class switch. This distinguishes CD57 from other activation markers like CD25 and CD80, which were homogeneously up- and downregulated on all blasts. In freshly isolated leukocytes from different tissues, only a small fraction of B cells was CD57<sup>+</sup>. However, more class-switched IgA<sup>+</sup> cells (up to 30%) expressed CD57 than unswitched cells and tissue dependent, more marker-negative/Ig<sup>+</sup> cells (up to 60%) than marker-positive/Ig<sup>+</sup> cells, which indicates a higher activation state in these subpopulations.

Analyzing CD57/chB6 expression by flow cytometry, Mast and colleagues identified three different B cell populations in spleen and cecal tonsil, describing them as CD57<sup>+</sup>/chB6<sup>high</sup> resting B cells, CD57<sup>+</sup>/chB6<sup>high</sup> GC B cells and CD57<sup>+</sup>/chB6<sup>low</sup> plasma cells (56). This classification was based the observation that immunohistochemistry showed weak CD57 staining in GCs and a lack of CD57 on B cells in the periellipsoidal white pulp. However, this interpretation of flow data is not supported by data. At least in the spleen, standard leukocyte preparations do not contain GC B cells as the encapsulated GCs stick to the artery tree (107) and are not included by regular isolation protocols. In addition, some chB6 expression can also be found on macrophages (108) but no further characteristics of the CD57<sup>+</sup>/chB6<sup>low</sup> population were described. Though we also found these populations, we think designation of these two populations as GC and plasma cells seems not really supported.

The observed, frequent CD57 expression on chicken B cells is in contrast to mammalian data and no parallels can be drawn from human and mouse B cells. In chickens, CD57 can be considered as a marker for a specific kind of B cell activation. Interestingly, bursal B cells, which proliferate and undergo BCR diversification, are all

CD57<sup>+</sup>, while the majority of blood B cells are negative. Hence, it seems justified to consider CD57<sup>+</sup> B cells as non-resting cells undergoing differentiation, though we currently cannot link its expression to a specific stage of B cell differentiation like GC, memory or plasma cells.

With the intent of getting plasma/memory cell specific markers, we generated two new mAbs directed against the chicken homologues of CD138 and TACI. Even though CD40L/IL-10 stimulation induced expression of TACI and CD138 on blasting cells, both molecules were downregulated post day two and only a very weak surface staining remained. Hence, in contrast to mice and humans (14, 16, 109) neither of them seems to be a marker for further differentiated B cells but rather indicates a form of B cells activation, like the processes which occur in GCs. This could also be an explanation for the very low frequency of CD138 or TACI positive cells in spleen and cecal tonsil as GC B cells are probably absent (spleen) or present in unknown numbers (CT). As only a fraction of B cell blasts becomes clearly CD138 and TACI positive, it will be interesting for future studies to characterize these positive and negative cells in more detail.

### 4.4 Improvement of the biphasic culture system

Surface marker and transcription factor analysis of differentiating B cells in our culture system revealed a biphasic process in CD40L/IL-10 stimulated mixed cultures of splenic leukocytes. We see an initial phase of cell activation (e.g. upregulation of CD25, CD80, TACI, CD138) followed by a second phase of increased cell differentiation (high *BLIMP-1* expression, loss of chB6 and BAFF-R, class switch). As mentioned above, this system was not optimized for final B cell differentiation. For cultures of both naïve and human GC B cells it was shown that termination of CD40L co-stimulation stops proliferation and promotes differentiation into antibody-secreting cells (110), while the continued presence of CD40L in GC cultures induced memory cells (46). Hence, in the future the removal of CD40L from the culture could help to induce further differentiation. In analogy to processes in mammalian GCs, addition of other cytokines could be necessary to generate more defined B cell phenotypes. A promising candidate is IL-21, which in mammalian GCs is produced by follicular T helper cells and strongly increases the percentage of CD38<sup>high</sup>/CD138<sup>high</sup> plasma cells as well as antibody production of CD40L activated human B cells (111). We cannot rule out that some IL-21 was already present in our mixed cultures as the large amount of proliferating T helper cells detected at later timepoints could be a source of IL-21.

Another cytokine, which is often included in mammalian B cell cultures is IL-2 (46, 112). The strong and rapid upregulation of CD25, the IL-2 receptor alpha chain, on CD40L/IL-10 stimulated B cells suggests that these chicken B cells may also respond to IL-2 and possibly, IL-2 was already present in the cultures: The emergency of a large T helper cell population in CD40L/IL-10 stimulated splenocyte cultures is unlikely to be a direct effect of



CD40L/IL-10, as IL-10 itself is known to suppress proliferation of T helper cells (113) instead of inducing it. However, it was shown that CD40 activated human and rabbit B cells can produce IL-2 (114, 115). If a similar reaction occurs in chicken B cells, this could be one possible explanation for the observed differentiation processes and cellular composition in our culture system. To identify the exact effects of individual cytokines, cultures of sort purified cells will be helpful in the future.

## 4.5 Age and antigen exposure

Though, we had not identified a specific marker for plasma or memory cells from analysis of *in vitro* stimulated B cells, the cultures had demonstrated that during the induced differentiation processes BCR surface expression, regardless of the Ig isotype, is not reduced and proven that BAFF-R and chB6 are lost during B cell differentiation. As all B cells, which emigrate from the bursa are chB6/BAFF-R positive, this allows the discrimination of marker-positive B cells from marker-negative B cells, which have undergone further differentiation. Based on these findings, we have examined the presence of marker-positive and marker-negative class switched cells under the influence of bursal involution and antigen exposure.

Loss of the bursa as primary B cell organ at the age of sexual maturity means a dramatic switch in the composition of the peripheral B cell pool. In birds with a productive bursa, about 60% of blood B cells are recent bursal emigrants with a half-life time of 30 hours (116), a population which misses post involution. In addition, frequent immunization and environmental antigen exposures add up over a chicken's lifetime, hence one could expect an increase in class-switched cells over time.

Surprisingly, bursa involution does not reduce the frequency of B cells in blood and spleen. In contrast, IgM<sup>+</sup> unswitched, marker<sup>+</sup> cells increased in both blood and spleen. We observed an increase in and marker-negative cells around the beginning of bursa involution (17w; IgA<sup>+</sup>) or in the middle of the laying period (27w; IgY<sup>+</sup>). However, this was no continuous change until the end of the laying period (70w) and can more likely be attributed to the vaccination scheme before the beginning of the laying period than an increase due to lifetime. Interestingly, in human blood, plasma cells represent age dependent around 1-2% of circulating B cells (60), which fits very well with our data and supports the staining strategy.

Another strong intervention into the B cell system was the application of a live IBDV vaccine, leading to B cell depletion in the bursa, which is followed by recovery of the organ (117). At the same time, the vaccine induces a strong anti-IBDV antibody response, which we thought could be associated with an increase of marker-negative and class-switched B cells. Four weeks post vaccination the proportion of the different fractions had completely changed, though it has to be considered that the B cell numbers were still quite low. Both, marker-positive and marker-negative class-switched as well as marker-negative unswitched IgM<sup>+</sup> cells, which are all correlates of differentiated cells, were significantly increased in vaccinated birds. This effect could be elicited either by an increased resistance of differentiated cells to IBDV infection or an

increased generation of new IBDV specific cells. To address the causation, further studies with the determination of absolute cells counts and the identification of IBDV specific cells would be helpful. As memory cells should increase after vaccinations, the observed increase in class-switched cells in this first experiment showed that a more detailed comparison of marker-positive and negative class-switched cells post vaccination can be a very promising approach.

Overall, we have for the first time shown, that IL-10 together with CD40L induces strong activation of B cells in cultures of total chicken splenocytes, which includes proliferation, Ig class switch and increased antibody secretion. Characterization of these cells revealed an early GC (CD80, CD25, MHC class II high) and a later plasmablast-like (loss of chB6/BAFF-R, CD25 and CXCR4) phenotype. Importantly, we found that BCR surface expression remains high during the observed differentiation processes. Based on this, we discriminated *in vivo* two populations of class-switched cells: chB6<sup>+</sup> and BAFF-R<sup>+</sup> marker-positive and likely plasma cells containing chB6<sup>-</sup> and BAFF-R<sup>-</sup> marker-negative cells, whose presence was less affected by age than by vaccination.

These results can now serve as a starting point to identify further differentiation factors *in vitro*, perform next generation sequencing of the cells for the identification of specific memory and plasma cell markers and evaluate the effects of different vaccines on their presence to obtain a better understanding into the chicken's antibody response.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The animal studies were approved by Government of Upper Bavaria, identification code: 55.2-1-54-2532.0-60-2015 for blood sampling and 55.21-1-54-2531.6-12-09 for the immunization of mice. The studies were conducted in accordance with the local legislation and institutional requirements.

## Author contributions

DLR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. MS: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. MK: Formal analysis, Investigation, Methodology, Writing – original draft. JT: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. BS: Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft. SR: Formal



analysis, Funding acquisition, Methodology, Resources, Writing – original draft. SH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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## References

- Sharma JM. Introduction to poultry vaccines and immunity. *Adv Vet Med.* (1999) 41:481–94. doi: 10.1016/S0065-3519(99)80036-6
- Swayne DE, Pavade G, Hamilton K, Vallat B, Miyagishima K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. *Rev Sci Tech.* (2011) 30:839–70. doi: 10.20506/rst.30.3.2081
- Glick B, Chang TS, Jaap RG. The bursa of fabricius and antibody production. *Poultry Sci.* (1956) 35:224–5. doi: 10.3382/ps.0350224
- Jeurissen SH, Janse EM. Germinal centers develop at predilected sites in the chicken spleen. *Adv Exp Med Biol.* (1994) 355:237–41. doi: 10.1007/978-1-4615-2492-2\_40
- Bellamy D, Mohamed K. A comparative study of age involution of the bursa of Fabricius an thymus in birds. *Thymus.* (1982) 4:107–14.
- Toivanen P, Toivanen A. Bursal and postbursal stem cells in chicken. *Funct characteristics. Eur J Immunol.* (1973) 3:585–95. doi: 10.1002/eji.1830030912
- Toivanen P, Toivanen A, Molnar G, Sorvari T. Bursal and postbursal cells in chicken: age-dependence of germinal center formation in spleen. *Int Arch Allergy Appl Immunol.* (1974) 47:749–61. doi: 10.1159/000231266
- Michael JH, Ratcliffe SH. B cells, the bursa of Fabricius, and the generation of antibody repertoires. In: Bernd Kaspers KAS, Göbel TW, Vervelde L, editors. *Avian immunology, 3 rd.* United Kingdom: Academic Press (2022). p. 71–99.
- Jeurissen SH. The role of various compartments in the chicken spleen during an antigen-specific humoral response. *Immunology.* (1993) 80:29–33.
- Savage ML, Olah I, Scott TR. Plasma cell proliferation in the chicken harderian gland. *Cell Proliferation basic Clin science.* (1992) 25:337–44. doi: 10.3382/ps.0721273
- Nagase F, Nakashima I, Kato N. Studies on the immune response in chickens. IV. Generation of hapten-specific memory and absence of increase in carrier-specific helper memory in antibody response to sheep red blood cell antigen and its hapten-conjugate. *Dev Comp Immunol.* (1983) 7:127–37. doi: 10.1016/0145-305X(83)90061-7
- Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker. *Immunol Today.* (2000) 21:204–6. doi: 10.1016/S0167-5699(00)01605-4
- Wijdenes J, Vooijs WC, Clement C, Post J, Morard F, Vita N, et al. A plasmacyte selective monoclonal antibody (B-B4) recognizes syndecan-1. *Br J Haematol.* (1996) 94:318–23. doi: 10.1046/j.1365-2141.1996.d01-1811.x
- Costes V, Magen V, Legouffe E, Durand L, Baldet P, Rossi JF, et al. The Mi15 monoclonal antibody (anti-syndecan-1) is a reliable marker for quantifying plasma

## Conflict of interest

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## Supplementary material

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- cells in paraffin-embedded bone marrow biopsy specimens. *Hum Pathol.* (1999) 30:1405–11. doi: 10.1016/S0046-8177(99)90160-0
- Castigli E, Wilson SA, Scott S, Dedeoglu F, Xu S, Lam KP, et al. TACI and BAFF-R mediate isotype switching in B cells. *J Exp Med.* (2005) 201:35–9. doi: 10.1084/jem.20032000
- Garcia-Carmona Y, Cols M, Ting AT, Radigan L, Yuk FJ, Zhang L, et al. Differential induction of plasma cells by isoforms of human TACI. *Blood.* (2015) 125:1749–58. doi: 10.1182/blood-2014-05-575845
- Garcia-Carmona Y, Fribourg M, Sowa A, Cerutti A, Cunningham-Rundles C. TACI and endogenous APRIL in B cell maturation. *Clin Immunol.* (2023) 253:109689. doi: 10.1016/j.clim.2023.109689
- Novak AJ, Darce JR, Arendt BK, Harder B, Henderson K, Kindsvogel W, et al. Expression of BCMA, TACI, and BAFF-R in multiple myeloma: a mechanism for growth and survival. *Blood.* (2004) 103:689–94. doi: 10.1182/blood-2003-06-2043
- O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med.* (2004) 199:91–8. doi: 10.1084/jem.20031330
- Tregaskes CA, Bumstead N, Davison TF, Young JR. Chicken B-cell marker chB6 (Bu-1) is a highly glycosylated protein of unique structure. *Immunogenetics.* (1996) 44:212–7. doi: 10.1007/BF02602587
- Houssaint E, Diez E, Pink JR. Ontogeny and tissue distribution of the chicken Bu-1a antigen. *Immunology.* (1987) 62:463–70. doi: 10.1002/eji.1830190204
- Houssaint E, Lassila O, Vainio O. Bu-1 antigen expression as a marker for B cell precursors in chicken embryos. *Eur J Immunol.* (1989) 19:239–43. doi: 10.1002/eji.1830190204
- Kothlow S, Morgenroth I, Tregaskes CA, Kaspers B, Young JR. CD40 ligand supports the long-term maintenance and differentiation of chicken B cells in culture. *Dev Comp Immunol.* (2008) 32:1015–26. doi: 10.1016/j.dci.2008.01.012
- Reddy SK, Hu T, Gudivada R, Staines KA, Wright KE, Vickstaff I, et al. The BAFF-Interacting receptors of chickens. *Dev Comp Immunol.* (2008) 32:1076–87. doi: 10.1016/j.dci.2008.02.004
- Schneider K, Kothlow S, Schneider P, Tardivel A, Göbel T, Kaspers B, et al. Chicken BAFF—a highly conserved cytokine that mediates B cell survival. *Int Immunol.* (2004) 16:139–48. doi: 10.1093/intimm/dxh015
- Kothlow S, Morgenroth I, Graef Y, Schneider K, Riehl I, Staeheli P, et al. Unique and conserved functions of B cell-activating factor of the TNF family (BAFF) in the chicken. *Int Immunol.* (2007) 19:203–15. doi: 10.1093/intimm/dxl137



27. Jax E, Werner E, Muller I, Schaerer B, Kohn M, Olofsson J, et al. Evaluating effects of AIV infection status on ducks using a flow cytometry-based differential blood count. *Microbiol Spectr.* (2023) 11:e0435122. doi: 10.1128/spectrum.04351-22
28. Kaiser P, Poh TY, Rothwell L, Avery S, Balu S, Pathania US, et al. A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res.* (2005) 25:467–84. doi: 10.1089/jir.2005.25.467
29. Liu YJ, Malisan F, de Bouteiller O, Guret C, Lebecque S, Banchereau J, et al. Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. *Immunity.* (1996) 4:241–50. doi: 10.1016/S1074-7613(00)80432-X
30. Dahan A, Reynaud CA, Weill JC. Nucleotide sequence of the constant region of a chicken mu heavy chain immunoglobulin mRNA. *Nucleic Acids Res.* (1983) 11:5381–9. doi: 10.1093/nar/11.16.5381
31. Parvari R, Avivi A, Lentner F, Ziv E, Tel-Or S, Burstein Y, et al. Chicken immunoglobulin gamma-heavy chains: limited VH gene repertoire, combinatorial diversification by D gene segments and evolution of the heavy chain locus. *EMBO J.* (1988) 7:739–44. doi: 10.1002/j.1460-2075.1988.tb02870.x
32. Mansikka A. Chicken IgA H chains. Implications concerning the evolution of H chain genes. *J Immunol.* (1992) 149:855–61. doi: 10.4049/jimmunol.149.3.855
33. Bengten E, Wilson M, Miller N, Clem LW, Pilstrom L, Warr GW. Immunoglobulin isotypes: structure, function, and genetics. *Curr Top Microbiol Immunol.* (2000) 248:189–219. doi: 10.1007/978-3-642-59674-2\_9
34. Jonard PP, Rambaud JC, Dive C, Vaerman JP, Galian A, Delacroix DL. Secretion of immunoglobulins and plasma proteins from the jejunal mucosa. Transport rate and origin of polymeric immunoglobulin A. *J Clin Invest.* (1984) 74:525–35. doi: 10.1172/JCI11450
35. Brandtzaeg P, Farstad IN, Johansen FE, Morton HC, Norderhaug IN, Yamanaka T. The B-cell system of human mucosae and exocrine glands. *Immunol Rev.* (1999) 171:45–87. doi: 10.1111/j.1600-065X.1999.tb01342.x
36. Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. The immune geography of IgA induction and function. *Mucosal Immunol.* (2008) 1:11–22. doi: 10.1038/mi.2007.6
37. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med.* (1998) 188:1679–89. doi: 10.1084/jem.188.9.1679
38. Fecteau JF, Cote G, Neron S. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. *J Immunol.* (2006) 177:3728–36. doi: 10.4049/jimmunol.177.6.3728
39. Tucci FA, Kitanovski S, Johansson P, Klein-Hitpass L, Kahraman A, Durig J, et al. Biased IGH VDJ gene repertoire and clonal expansions in B cells of chronically hepatitis C virus-infected individuals. *Blood.* (2018) 131:546–57. doi: 10.1182/blood-2017-09-805762
40. Ivanyi J, Hudson L. Allelic exclusion of M1 (IgM) allotype on the surface of chicken B cells. *Immunology.* (1978) 35:941–5.
41. Schusser B, Collarini EJ, Pedersen D, Yi H, Ching K, Izquierdo S, et al. Expression of heavy chain-only antibodies can support B-cell development in light chain knockout chickens. *Eur J Immunol.* (2016) 46:2137–48. doi: 10.1002/eji.201546171
42. Mills DM, Cambier JC. B lymphocyte activation during cognate interactions with CD4+ T lymphocytes: molecular dynamics and immunologic consequences. *Semin Immunol.* (2003) 15:325–9. doi: 10.1016/j.smim.2003.09.004
43. Armitage RJ, Macduff BM, Spriggs MK, Fanslow WC. Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. *J Immunol.* (1993) 150:3671–80. doi: 10.4049/jimmunol.150.9.3671
44. Granato A, Hayashi EA, Baptista BJ, Bellio M, Nobrega A. IL-4 regulates Bim expression and promotes B cell maturation in synergy with BAFF conferring resistance to cell death at negative selection checkpoints. *J Immunol.* (2014) 192:5761–75. doi: 10.4049/jimmunol.1300749
45. Tosato G, Seamon KB, Goldman ND, Sehgal PB, May LT, Washington GC, et al. Monocyte-derived human B-cell growth factor identified as interferon-beta 2 (BSF-2, IL-6). *Science.* (1988) 239:502–4. doi: 10.1126/science.239.4839.502
46. Arpin C, Dechanet J, Van Kooten C, Merville P, Grouard G, Briere F, et al. Generation of memory B cells and plasma cells in vitro. *Science.* (1995) 268:720–2. doi: 10.1126/science.7537388
47. Yoon SO, Zhang X, Berner P, Choi YS. IL-21 and IL-10 have redundant roles but differential capacities at different stages of Plasma Cell generation from human Germinal Center B cells. *J Leukoc Biol.* (2009) 86:1311–8. doi: 10.1189/jlb.0409268
48. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol.* (2005) 175:7867–79. doi: 10.4049/jimmunol.175.12.7867
49. Naito M, Hainz U, Burkhardt UE, Fu B, Aho D, Stevenson KE, et al. CD40L-Tri, a novel formulation of recombinant human CD40L that effectively activates B cells. *Cancer Immunol Immunother.* (2013) 62:347–57. doi: 10.1007/s00262-012-1331-4
50. Malisan F, Briere F, Bridon JM, Harindranath N, Mills FC, Max EE, et al. Interleukin-10 induces immunoglobulin G isotype switch recombination in human CD40-activated naive B lymphocytes. *J Exp Med.* (1996) 183:937–47. doi: 10.1084/jem.183.3.937
51. Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, et al. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. *J Immunol.* (2004) 173:2675–82. doi: 10.4049/jimmunol.173.4.2675
52. Tregaskes CA, Glansbeek HL, Gill AC, Hunt LG, Burnside J, Young JR. Conservation of biological properties of the CD40 ligand, CD154 in a non-mammalian vertebrate. *Dev Comp Immunol.* (2005) 29:361–74. doi: 10.1016/j.dci.2004.09.001
53. Van Meter R, Good RA, Cooper MD. A 44 kilodalton cell surface homodimer regulates interleukin 2 production by activated human T lymphocytes. *J Immunol.* (1969) 102:370–4.
54. Leslie GA, Clem LW. Chicken immunoglobulins: biological half-lives and normal adult serum concentrations of IgM and IgY. *Proc Soc Exp Biol Med.* (1970) 134:195–8. doi: 10.3181/00379727-134-34758
55. Lebacqz-Verheyden AM, Vaerman JP, Heremans JF. Quantification and distribution of chicken immunoglobulins IgA, IgM and IgG in serum and secretions. *Immunology.* (1974) 27:683–92.
56. Mast J, Goddeeris BM. CD57, a marker for B-cell activation and splenic ellipsoid-associated reticular cells of the chicken. *Cell Tissue Res.* (1998) 291:107–15. doi: 10.1007/s004410050984
57. Rautenschlein S, Kraemer C, Vanmarcke J, Montiel E. Protective efficacy of intermediate and intermediate plus infectious bursal disease virus (IBDV) vaccines against very virulent IBDV in commercial broilers. *Avian Dis.* (2005) 49:231–7. doi: 10.1637/7310-112204R
58. Blanco E, Perez-Andres M, Arriba-Mendez S, Contreras-Sanfeliciano T, Criado I, Pelak O, et al. Age-associated distribution of normal B-cell and plasma cell subsets in peripheral blood. *J Allergy Clin Immunol.* (2018) 141:2208–19 e16. doi: 10.1016/j.jaci.2018.02.017
59. Budeus B, Kibler A, Kuppers R. Human IgM-expressing memory B cells. *Front Immunol.* (2023) 14:1308378. doi: 10.3389/fimmu.2023.1308378
60. Caraux A, Klein B, Paiva B, Bret C, Schmitz A, Fuhler GM, et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. *Haematologica.* (2010) 95:1016–20. doi: 10.3324/haematol.2009.018689
61. Jeurissen SH, Janse EM, Koch G, De Boer GF. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res.* (1989) 258:119–24. doi: 10.1007/BF00223151
62. Honjo K, Hagiwara T, Itoh K, Takahashi E, Hirota Y. Immunohistochemical analysis of tissue distribution of B and T cells in germfree and conventional chickens. *J Vet Med Sci.* (1993) 55:1031–4. doi: 10.1292/jvms.55.1031
63. Mcmillan R, Yelenosky R, Heath V, Longmire RL, Lang JE, Craddock CG. Immunoglobulin-synthesis by human lymphoid tissues - normal bone-marrow as a major site of igg production. *J Immunol.* (1972) 109:1386. doi: 10.4049/jimmunol.109.6.1386
64. Manz RA, Radbruch A. Plasma cells for a lifetime? *Eur J Immunol.* (2002) 32:923–7. doi: 10.1002/1521-4141(200204)32:4<923::AID-IMMU923>3.0.CO;2-1
65. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, et al. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol.* (2003) 171:1684–90. doi: 10.4049/jimmunol.171.4.1684
66. de Geus ED, Rebel JM, Vervelde L. Kinetics of the avian influenza-specific humoral responses in lung are indicative of local antibody production. *Dev Comp Immunol.* (2012) 36:317–22. doi: 10.1016/j.dci.2011.05.005
67. Terstappen LW, Johnsen S, Segers-Nolten IM, Loken MR. Identification and characterization of plasma cells in normal human bone marrow by high-resolution flow cytometry. *Blood.* (1990) 76:1739–47. doi: 10.1182/blood.V76.9.1739.1739
68. Mei HE, Wirries I, Frolich D, Brissler M, Giesecke C, Grun JR, et al. A unique population of IgG-expressing plasma cells lacking CD19 is enriched in human bone marrow. *Blood.* (2015) 125:1739–48. doi: 10.1182/blood-2014-02-555169
69. Briere F, Servet-Delprat C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin 10 induces naive surface immunoglobulin D+ (slgD+) B cells to secrete IgG1 and IgG3. *J Exp Med.* (1994) 179:757–62. doi: 10.1084/jem.179.2.757
70. Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity.* (2002) 17:51–62. doi: 10.1016/S1074-7613(02)00335-7
71. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity.* (2003) 19:607–20. doi: 10.1016/S1074-7613(03)00267-X
72. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell.* (2000) 102:553–63. doi: 10.1016/S0092-8674(00)00078-7
73. Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell.* (2000) 102:565–75. doi: 10.1016/S0092-8674(00)00079-9
74. Nossal GJ, Pike BL. Differentiation of B lymphocytes from stem cell precursors. *Adv Exp Med Biol.* (1973) 29:11–8. doi: 10.1007/978-1-4615-9017-0\_3
75. Tyler BM, Cowman AF, Gerondakis SD, Adams JM, Bernard O. mRNA for surface immunoglobulin gamma chains encodes a highly conserved transmembrane sequence and a 28-residue intracellular domain. *Proc Natl Acad Sci U S A.* (1982) 79:2008–12. doi: 10.1073/pnas.79.6.2008



76. Sitia R, Neuberger MS, Milstein C. Regulation of membrane IgM expression in secretory B cells: translational and post-translational events. *EMBO J.* (1987) 6:3969–77. doi: 10.1002/j.1460-2075.1987.tb02739.x
77. Pinto D, Montani E, Bolli M, Garavaglia G, Sallusto F, Lanzavecchia A, et al. A functional BCR in human IgA and IgM plasma cells. *Blood.* (2013) 121:4110–4. doi: 10.1182/blood-2012-09-459289
78. Blanc P, Moro-Sibilot L, Barthly L, Jagot F, This S, de Bernard S, et al. Mature IgM-expressing plasma cells sense antigen and develop competence for cytokine production upon antigenic challenge. *Nat Commun.* (2016) 7:13600. doi: 10.1038/ncomms13600
79. Jourdan M, Caraux A, De Vos J, Fiol G, Larroque M, Cognot C, et al. An *in vitro* model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. *Blood.* (2009) 114:5173–81. doi: 10.1182/blood-2009-07-235960
80. Koike T, Fujii K, Kometani K, Butler NS, Funakoshi K, Yari S, et al. Progressive differentiation toward the long-lived plasma cell compartment in the bone marrow. *J Exp Med.* (2023) 220. doi: 10.1084/jem.2022117
81. Darce JR, Arendt BK, Wu X, Jelinek DF. Regulated expression of BAFF-binding receptors during human B cell differentiation. *J Immunol.* (2007) 179:7276–86. doi: 10.4049/jimmunol.179.11.7276
82. Muller-Winkler J, Mitter R, Rappe JCF, Vanes L, Schweighoffer E, Mohammadi H, et al. Critical requirement for BCR, BAFF, and BAFFR in memory B cell survival. *J Exp Med.* (2021) 218. doi: 10.1084/jem.20191393
83. Iisu BL, Harless SM, Lindsley RC, Hilbert DM, Cancro MP. Cutting edge: BlyS enables survival of transitional and mature B cells through distinct mediators. *J Immunol.* (2002) 168:5993–6. doi: 10.4049/jimmunol.168.12.5993
84. Sims GP, Ettinger R, Shirota Y, Yarbboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. *Blood.* (2005) 105:4390–8. doi: 10.1182/blood-2004-11-4284
85. Dora D, Fejszak N, Goldstein AM, Minko K, Nagy N. Ontogeny of ramified CD45 cells in chicken embryo and their contribution to bursal secretory dendritic cells. *Cell Tissue Res.* (2017) 368:353–70. doi: 10.1007/s00441-017-2595-y
86. Nagy N, Busalt F, Halasy V, Kohn M, Schmieder S, Fejszak N, et al. In and out of the bursa—the role of CXCR4 in chicken B cell development. *Front Immunol.* (2020) 11:1468. doi: 10.3389/fimmu.2020.01468
87. Amu S, Tarkowski A, Dörner T, Bokarewa M, Brisslert M. The human immunomodulatory CD25+ B cell population belongs to the memory B cell pool. *Scand J Immunol.* (2007) 66:77–86. doi: 10.1111/j.1365-3083.2007.01946.x
88. Inaba A, Tuong ZK, Zhao TX, Stewart AP, Mathews R, Truman L, et al. Low-dose IL-2 enhances the generation of IL-10-producing immunoregulatory B cells. *Nat Commun.* (2023) 14:2071. doi: 10.1038/s41467-023-37424-w
89. Guzman-Rojas L, Sims-Mourtada JC, Rangel R, Martinez-Valdez H. Life and death within germinal centres: a double-edged sword. *Immunology.* (2002) 107:167–75. doi: 10.1046/j.1365-2567.2002.01494.x
90. Klein U, Tu Y, Stolovitzky GA, Keller JL, Haddad J Jr., Miljkovic V, et al. Gene expression dynamics during germinal center transit in B cells. *Ann N Y Acad Sci.* (2003) 987:166–72. doi: 10.1111/j.1749-6632.2003.tb06045.x
91. Good-Jacobson KL, Song E, Anderson S, Sharpe AH, Shlomchik MJ. CD80 expression on B cells regulates murine T follicular helper development, germinal center B cell survival, and plasma cell generation. *J Immunol.* (2012) 188:4217–25. doi: 10.4049/jimmunol.1102885
92. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol.* (2004) 5:943–52. doi: 10.1038/ni1100
93. Bannard O, Horton RM, Allen CD, An J, Nagasawa T, Cyster JG. Germinal center centroblasts transition to a centrocyte phenotype according to a timed program and depend on the dark zone for effective selection. *Immunity.* (2013) 39:912–24. doi: 10.1016/j.immuni.2013.08.038
94. Weber TS. Cell cycle-associated CXCR4 expression in germinal center B cells and its implications on affinity maturation. *Front Immunol.* (2018) 9:1313. doi: 10.3389/fimmu.2018.01313
95. Abo T, Balch CM. A differentiation antigen of human nk-cells and K-cells identified by a monoclonal-antibody (Hnk-1). *J Immunol.* (1981) 127:1024–9. doi: 10.4049/jimmunol.127.3.1024
96. Schuller-Petrovic S, Gebhart W, Lassmann H, Rumpold H, Kraft D. A shared antigenic determinant between natural killer cells and nervous tissue. *Nature.* (1983) 306:179–81. doi: 10.1038/306179a0
97. Lipinski M, Braham K, Caillaud JM, Carlu C, Tursz T. HNK-1 antibody detects an antigen expressed on neuroectodermal cells. *J Exp Med.* (1983) 158:1775–80. doi: 10.1084/jem.158.5.1775
98. Krishnaraj R, Svanborg A. Preferential accumulation of mature NK cells during human immunosenescence. *J Cell Biochem.* (1992) 50:386–91. doi: 10.1002/jcb.240500407
99. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood.* (2010) 116:3865–74. doi: 10.1182/blood-2010-04-282301
100. d'Angeac AD, Monier S, Pilling D, Travaglio-Encinoza A, Reme T, Salmon M. CD57+ T lymphocytes are derived from CD57- precursors by differentiation occurring in late immune responses. *Eur J Immunol.* (1994) 24:1503–11. doi: 10.1002/eji.1830240707
101. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood.* (2003) 101:2711–20. doi: 10.1182/blood-2002-07-2103
102. Chou DKH, Ilyas AA, Evans JE, Costello C, Quarles RH, Jungalwala FB. Structure of sulfated glucuronyl glycolipids in the nervous-system reacting with hnk-1 antibody and some igm paraproteins in neuropathy. *J Biol Chem.* (1986) 261:1717–25. doi: 10.1016/S0021-9258(18)67303-X
103. Voshol H, van Zuylen CW, Orberger G, Vliegthart JF, Schachner M. Structure of the HNK-1 carbohydrate epitope on bovine peripheral myelin glycoprotein P0. *J Biol Chem.* (1996) 271:22957–60. doi: 10.1074/jbc.271.38.22957
104. Ariga T, Kohriyama T, Fredo L, Latov N, Saito M, Kon K, et al. Characterization of sulfated glucuronic-acid containing glycolipids reacting with igm M-proteins in patients with neuropathy. *J Biol Chem.* (1987) 262:848–53. doi: 10.1016/S0021-9258(19)75864-5
105. Wattang E NL, Juul-Madsen HR, Dalgaard TS. Preliminary characterisation of Cd107a and Cd57 as potential activation markers of chicken cytotoxic T-cells. In: *XII AIRG International Avian Immunology Research Group Meeting*. Edinburgh, UK (2012).
106. Ramskold D, Parodis I, Lakshminanth T, Sippl N, Khademi M, Chen Y, et al. B cell alterations during BAFF inhibition with belimumab in SLE. *EBioMedicine.* (2019) 40:517–27. doi: 10.1016/j.ebiom.2018.12.035
107. Yasuda M, Kajiwa E, Ekino S, Taura Y, Hirota Y, Horiuchi H, et al. Immunobiology of chicken germinal center: I. Changes in surface Ig class expression in the chicken splenic germinal center after antigenic stimulation. *Dev Comp Immunol.* (2003) 27:159–66. doi: 10.1016/S0145-305X(02)00066-6
108. Hartle S, Sutton K, Vervelde L, Dalgaard TS. Delineation of chicken immune markers in the era of omics and multicolor flow cytometry. *Front Vet Sci.* (2024) 11:1385400. doi: 10.3389/fvets.2024.1385400
109. Good KL, Avery DT, Tangye SG. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *J Immunol.* (2009) 182:890–901. doi: 10.4049/jimmunol.182.2.890
110. Marsman C, Verstegen NJ, Streutker M, Jorritsma T, Boon L, Ten Brinke A, et al. Termination of CD40L co-stimulation promotes human B cell differentiation into antibody-secreting cells. *Eur J Immunol.* (2022) 52:1662–75. doi: 10.1002/eji.202249972
111. Possamai D, Page G, Panes R, Gagnon F, Lapointe R. CD40L-Stimulated B Lymphocytes Are Polarized toward APC Functions after Exposure to IL-4 and IL-21. *J Immunol.* (2021) 207:77–89. doi: 10.4049/jimmunol.2001173
112. Hipp N, Symington H, Pastoret C, Caron G, Monvoisin C, Tarte K, et al. IL-2 imprints human naive B cell fate towards plasma cell through ERK/ELK1-mediated BACH2 repression. *Nat Commun.* (2017) 8:1443. doi: 10.1038/s41467-017-01475-7
113. Taga K, Tosato G. IL-10 inhibits human T-cell proliferation and il-2 production. *J Immunol.* (1992) 148:1143–8. doi: 10.4049/jimmunol.148.4.1143
114. Lagoo A, Tseng CK, Sell S. Interleukin 2 produced by activated B lymphocytes acts as an autocrine proliferation-inducing lymphokine. *Cytokine.* (1990) 2:272–9. doi: 10.1016/1043-4666(90)90028-R
115. Kindler V, Matthes T, Jeannin P, Zubler RH. Interleukin-2 secretion by human B lymphocytes occurs as a late event and requires additional stimulation after CD40 cross-linking. *Eur J Immunol.* (1995) 25:1239–43. doi: 10.1002/eji.1830250516
116. Paramithiotis E, Ratcliffe MJ. Bursa-dependent subpopulations of peripheral B lymphocytes in chicken blood. *Eur J Immunol.* (1993) 23:96–102. doi: 10.1002/eji.1830230116
117. Dobner M, Auerbach M, Mundt E, Icken W, Rautenschlein S. Genotype-associated differences in bursal recovery after infectious bursal disease virus (IBDV) inoculation. *Vet Immunol Immunopathol.* (2020) 220:109993. doi: 10.1016/j.vetimm.2019.109993
118. Gobel TW, Kaspers B, Stangassinger M. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. *Int Immunol.* (2001) 13:757–62. doi: 10.1093/intimm/13.6.757
119. Arstila TP, Vainio O, Lassila O. Evolutionarily conserved function of CD28 in alpha beta T cell activation. *Scand J Immunol.* (1994) 40:368–71. doi: 10.1111/j.1365-3083.1994.tb03476.x
120. Norup LR, Dalgaard TS, Pedersen AR, Juul-Madsen HR. Assessment of Newcastle disease-specific T cell proliferation in different inbred MHC chicken lines. *Scand J Immunol.* (2011) 74:23–30. doi: 10.1111/j.1365-3083.2011.02534.x
121. Escot S, Blavet C, Hartle S, Duband JL, Fournier-Thibault C. Misregulation of SDF1-CXCR4 signaling impairs early cardiac neural crest cell migration leading to conotruncal defects. *Circ Res.* (2013) 113:505–16. doi: 10.1161/CIRCRESAHA.113.301333
122. Haertle S, Alzuheir I, Busalt F, Waters V, Kaiser P, Kaufer BB. Identification of the receptor and cellular ortholog of the marek's disease virus (MDV) CXC chemokine. *Front Microbiol.* (2017) 8:2543. doi: 10.3389/fmicb.2017.02543
123. Erhard MH, Von Quistorp I, Schraner I, Jungling A, Kaspers B, Schmidt P, et al. Development of specific enzyme-linked immunosorbent antibody assay systems for the detection of chicken immunoglobulins G, M, and A using monoclonal antibodies. *Poult Sci.* (1992) 71:302–10. doi: 10.3382/ps.0710302



## **4.2 Supplementary Material**

Im Folgenden ist das Supplementary Material zur Publikation “Characterization of class-switched B cells in Chickens”, erschienen am 21.11.2024 in Frontiers in Immunology, Section B cell Biology, Volume 15 – 2024, wiedergegeben.



## Supplementary Material

### 1 Supplementary Tables

#### Supplementary Table 1

**Table S1.** Frequencies of sIg<sup>+</sup>/BAFF-R<sup>-</sup> and sIg<sup>+</sup>/BAFF-R<sup>+</sup> leukocytes from different lymphoid organs based on Figure 1 (mean  $\pm$  SDM; n=5)

|                      | IgM             |                  | IgA             |                 | IgY             |                 |
|----------------------|-----------------|------------------|-----------------|-----------------|-----------------|-----------------|
|                      | BAFF-R -        | BAFF-R +         | BAFF-R -        | BAFF-R +        | BAFF-R -        | BAFF-R +        |
| <b>PBLs</b>          | 0.20 $\pm$ 0.14 | 19.00 $\pm$ 4.55 | 0.20 $\pm$ 0.08 | 0.64 $\pm$ 0.37 | 0.09 $\pm$ 0.07 | 0.18 $\pm$ 0.10 |
| <b>Spleen</b>        | 0.13 $\pm$ 0.05 | 32.18 $\pm$ 5.80 | 0.10 $\pm$ 0.07 | 0.48 $\pm$ 0.21 | 0.07 $\pm$ 0.06 | 0.15 $\pm$ 0.08 |
| <b>Caecal tonsil</b> | 0.11 $\pm$ 0.07 | 51 $\pm$ 9.90    | 0.48 $\pm$ 0.17 | 7.18 $\pm$ 0.89 | 0.07 $\pm$ 0.09 | 0.15 $\pm$ 0.05 |
| <b>Bone marrow</b>   | 0.12 $\pm$ 0.06 | 19.25 $\pm$ 6.99 | 0.05 $\pm$ 0.04 | 0.33 $\pm$ 0.17 | 0.10 $\pm$ 0.07 | 0.06 $\pm$ 0.04 |

#### Supplementary Table 2

**Table S2.** Leukocytes from different lymphoid organs were stained for CD57 and the respective surface Ig (IgM, IgA or IgY). Frequencies of the different subpopulations shown in Figure 7 (mean  $\pm$  SD; n=5)

|                      | IgM             |                |                 | IgA             |                 |                | IgY             |                |                |
|----------------------|-----------------|----------------|-----------------|-----------------|-----------------|----------------|-----------------|----------------|----------------|
|                      | CD57+/ Ig-      | CD57+/Ig+      | CD57-/Ig+       | CD57+/ Ig-      | CD57+/Ig+       | CD57-/Ig+      | CD57+ Ig-       | CD57+/Ig+      | CD57-/Ig+      |
| <b>PBL</b>           | 40,95 $\pm$ 5,7 | 1,49 $\pm$ 0,9 | 19,35 $\pm$ 4,9 | 41,45 $\pm$ 3,5 | 0,47 $\pm$ 0,1  | 0,96 $\pm$ 0,3 | 35,98 $\pm$ 3,7 | 0,32 $\pm$ 0,4 | 0,46 $\pm$ 0,1 |
| <b>spleen</b>        | 20,68 $\pm$ 4,8 | 1,35 $\pm$ 0,5 | 32,28 $\pm$ 5,8 | 23,80 $\pm$ 6,4 | 0,16 $\pm$ 0,1  | 0,72 $\pm$ 0,1 | 22,5 $\pm$ 7,7  | 0,12 $\pm$ 0,1 | 0,35 $\pm$ 0,2 |
| <b>Caecal tonsil</b> | 21,05 $\pm$ 2,7 | 2,74 $\pm$ 1,3 | 47,48 $\pm$ 8,9 | 23,18 $\pm$ 2,8 | 2,33 $\pm$ 0,6  | 6,54 $\pm$ 1   | 23,45 $\pm$ 2,2 | 0,43 $\pm$ 0,1 | 0,86 $\pm$ 0,4 |
| <b>BM</b>            | 23,59 $\pm$ 2,3 | 1,15 $\pm$ 0,5 | 19,35 $\pm$ 5,8 | 23,23 $\pm$ 5,1 | 0,19 $\pm$ 0,03 | 0,51 $\pm$ 0,2 | 22,68 $\pm$ 4,7 | 0,09 $\pm$ 0,1 | 0,23 $\pm$ 0,2 |

## Supplementary Material

**Supplementary Table 3**

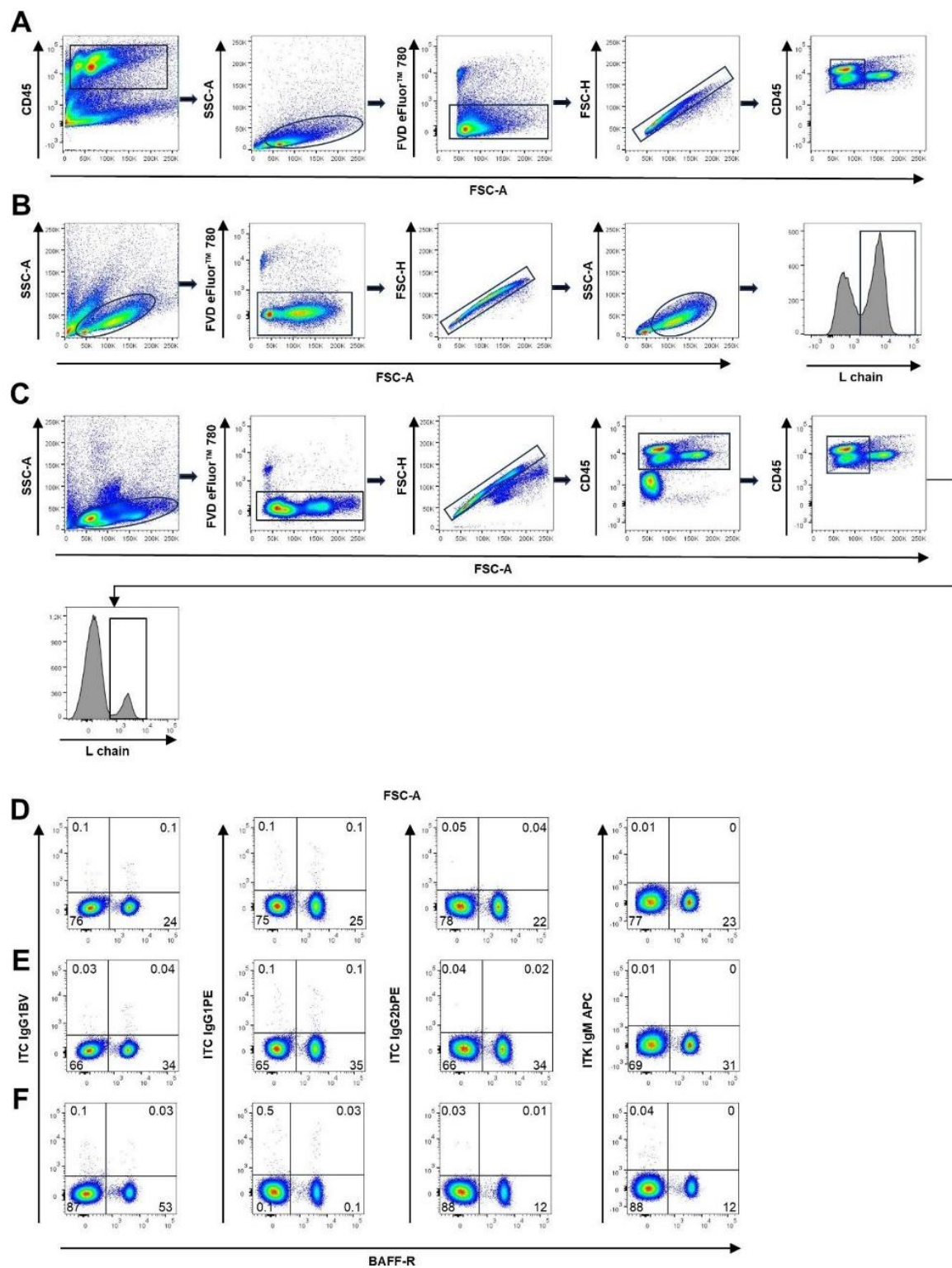
**Table S3.** Proportion of CD57<sup>+</sup> cells among the Ig<sup>+</sup> cells from different lymphoid organs based on the staining in Figure 6; (mean  $\pm$  SD; n=5).

|                      | <b>IgM</b>    | <b>IgA</b>      | <b>IgY</b>      |
|----------------------|---------------|-----------------|-----------------|
| <b>PBL</b>           | 7,1 $\pm$ 3,3 | 33,4 $\pm$ 3,9  | 29,2 $\pm$ 19,9 |
| <b>spleen</b>        | 4,1 $\pm$ 1,6 | 19,0 $\pm$ 7,9  | 21,4 $\pm$ 8,8  |
| <b>Caecal tonsil</b> | 5,2 $\pm$ 1,3 | 26,3 $\pm$ 5,9  | 34,9 $\pm$ 5,8  |
| <b>BM</b>            | 5,8 $\pm$ 2,2 | 29,2 $\pm$ 10,4 | 25,0 $\pm$ 6,9  |

## Supplementary Material

## 2 Supplementary Figures

## Supplementary Figure 1



## Supplementary Material

**Figure S1 Gating Strategies and Isotype controls**

In general, gating on CD45-positive cells was applied when analyzing different lymphoid organs (Figure 1 and 7). This approach was used because isolated cells from certain organs, particularly the bone marrow, typically contain a large proportion of non-leukocytes.

As splenocyte preparations usually consist almost exclusively from leukocytes, CD45 staining was omitted with the exception of IBDV vaccinated birds (Fig. 9) as the strong reaction to the applied live vaccines often leads to a higher content of nucleated erythrocytes in leukocyte preparations from spleen and blood.

As we could show that during differentiation B remain L chain positive, additional gating for L chain positive cells was applied to phenotyping experiments of splenocyte cultures.

(A) Gating strategy employed for Figure 1. Initially, a gate was established for CD45-positive cells, followed by subsequent gating for leukocytes in the scatter plot, exclusion of dead cells, and finally, gating for single cells. For PBMCs, an additional exclusion step for monocytes by size was included.

(B) Demonstrates the gating strategy utilized for splenic cell culture. Initially, the gate was set for leukocytes, followed by exclusion of dead cells and gating for single cells. Subsequently, single cells were further categorized into small and proliferating cells. Within the proliferating cell population, an additional gate was established for light chain-positive cells. This gating strategy is depicted on cells stimulated with CD40L and IL-10 after 96 hours of stimulation. For the assessment of unstimulated splenic leukocytes, the fourth step of this strategy was omitted, as no proliferating cell population was observed.

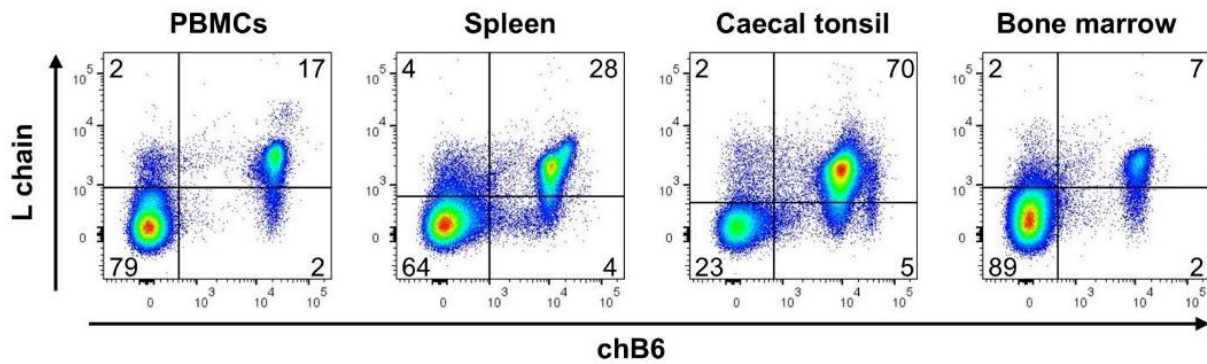
(C) Depicts the gating used in the vaccination trial (Fig. 9). The first gate was set on leukocytes followed by dead cell exclusion, single cell gating, and exclusion of thrombocytes by their lower expression of CD45. Monocytes were excluded via cell size. Additional gating for light chain-positive cells was added when needed.

Isotype controls for sIg staining (IgG1, IgG2b) and CD57 (IgM) staining are displayed for bone marrow (D), spleen (E), and PBMCs (F). Blots are gated for viable single cells.



## Supplementary Material

## Supplementary Figure 2

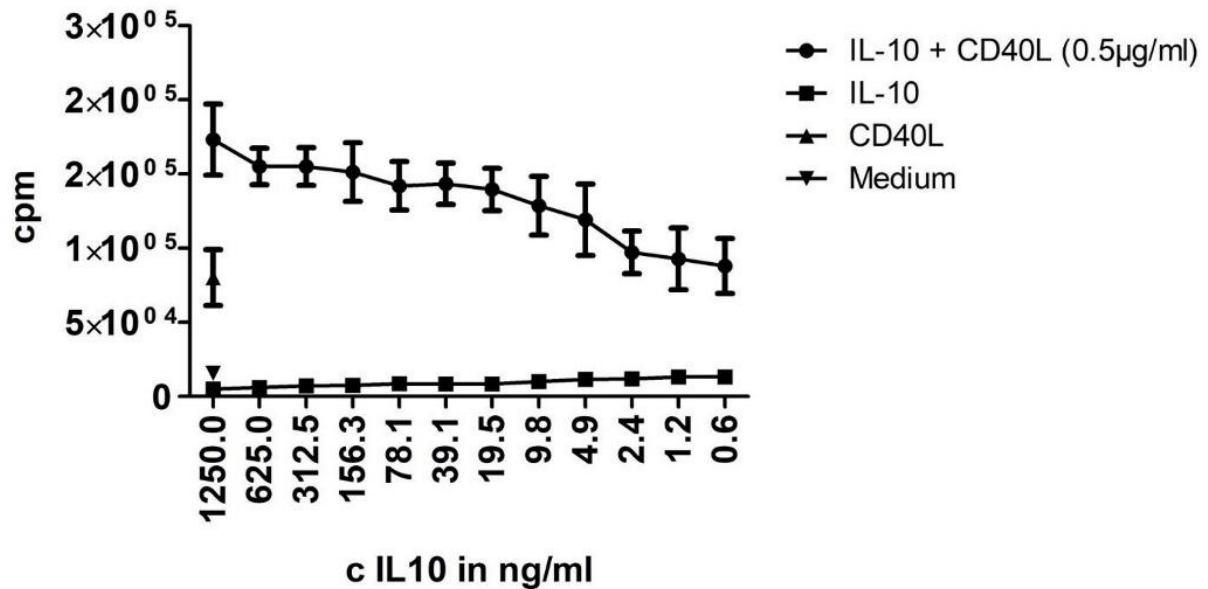
**Figure S2 L chain expression against chB6**

Leukocytes from the respective tissues of a young adult chicken (5,5 month) were stained with antibodies for L chain and chB6 and analyzed by flow cytometry.

Blots were gated for viable single lymphocytes. Data are representative for four independent experiments.

## Supplementary Material

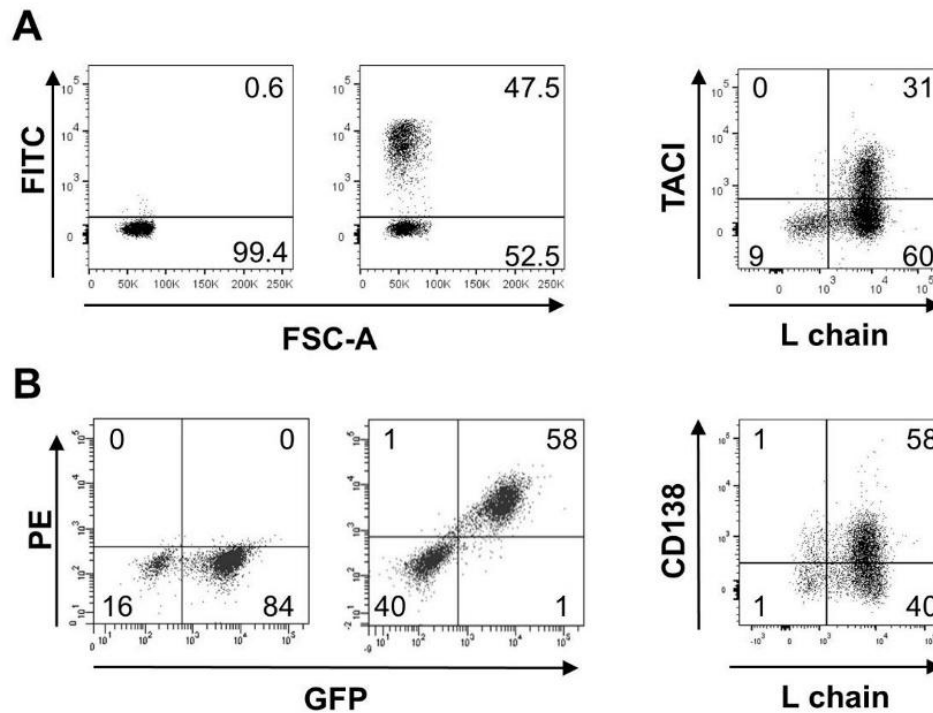
## Supplementary Figure 3

**Figure S3 Dose dependent effect of IL-10**

Leukocytes were isolated from spleen and incubated for 48h in the presence of CD40L, IL-10, a combination of both cytokines as well as medium only. In the combined stimulation, CD40L concentration was kept constant at 0,5μg/ml, while IL10 was serially diluted. After 16h cells were labeled with [<sup>3</sup>H]-Thymidine and harvested 24h later. Shown are mean ± SD of three experiments.

## Supplementary Material

## Supplementary Figure 4

**Figure S4 Clone testing on transfected 293 cells**

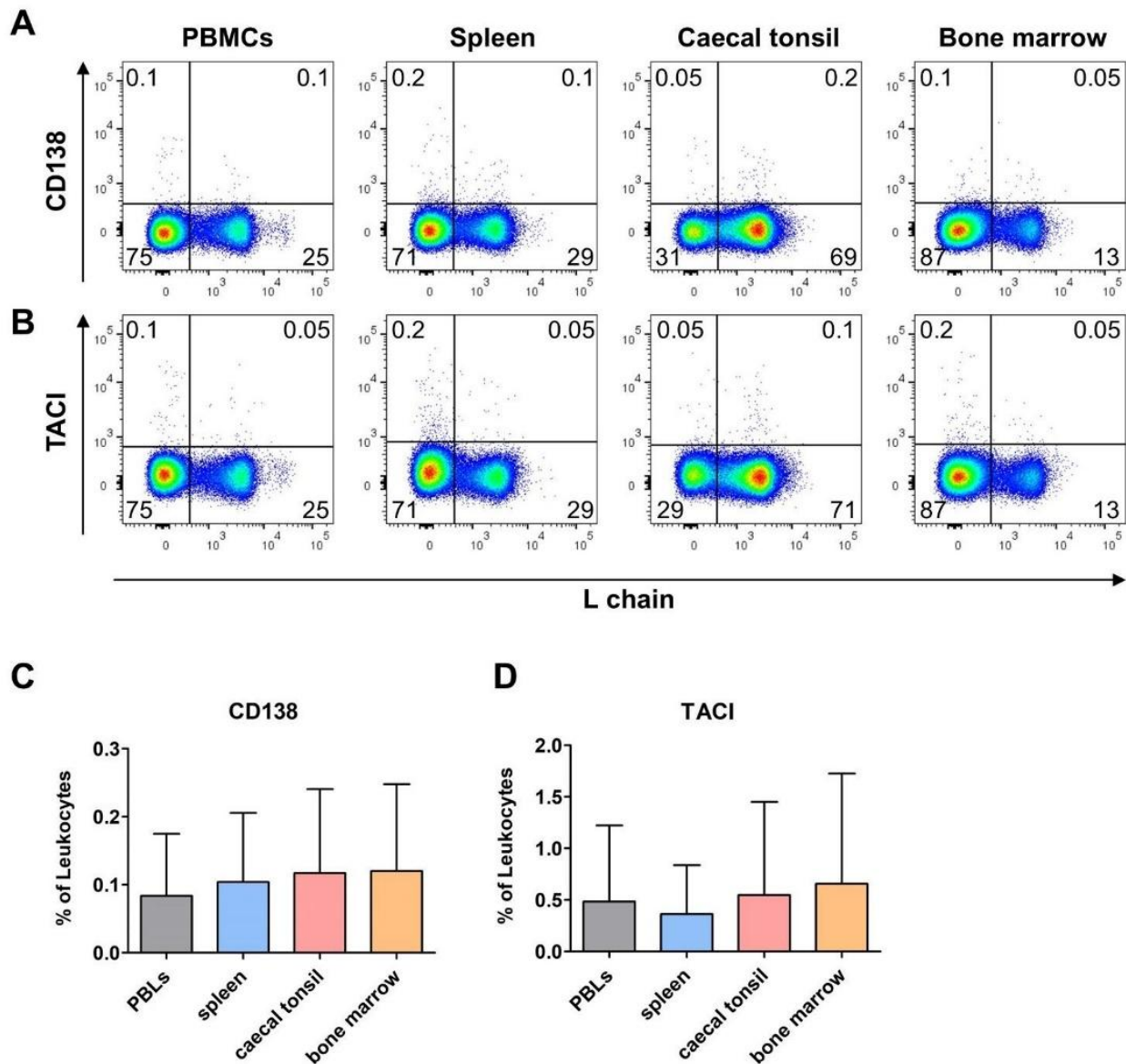
(A) Illustrates the evaluation of the preselected TACI clone on stably transfected 293 cells; with the negative control depicted on the left and the preselected clone tested on a mixture of 293 and stably transfected 293-TACI cells. The dot blot on the right displays the staining of the selected clone on splenic leukocytes stimulated for 48 hours.

(B) Illustrates the testing of the preselected CD138 antibody clone on a mixture of 293-chCD138GFP cells with untransfected 293 cells, with the negative control shown on the left. The dot blot on the right displays the staining of the selected clone on splenic leukocytes stimulated for 48 hours.

Blots are gated for viable single cells.

## Supplementary Material

## Supplementary Figure 5

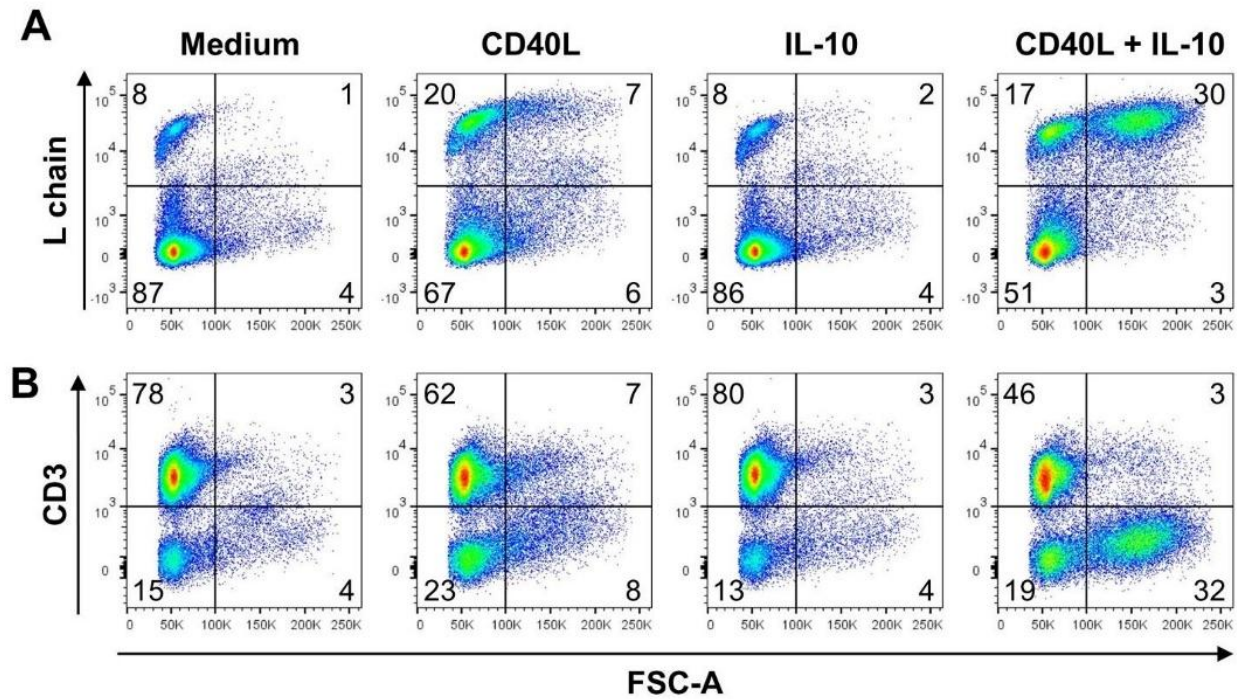
**Figure S5: TACI and CD138 expression in leukocytes**

Blots exemplify the expression of chCD138 (A) and chTACI (B) on CD45<sup>+</sup> viable single cells isolated from different organs.

Bar graphs depict the frequency of chCD138 (C) and chTACI (D) on CD45<sup>+</sup> viable single cells. Shown are mean  $\pm$  SD of four experiments.

## Supplementary Material

## Supplementary Figure 6

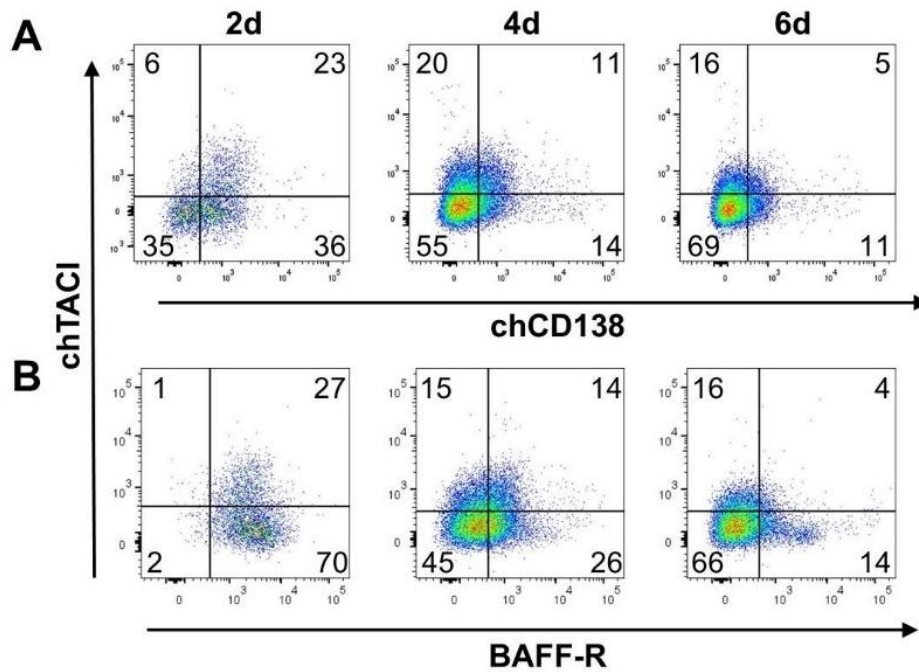
**Figure S6 Blast induction by different cytokines**

Depicts live single cells of 72h stimulated splenic leukocytes (shown in Figure 1A). Size of the cells is shown against their surface light-chain (A) and CD3 (B) expression. Plots are gated for viable single cells.



## Supplementary Material

## Supplementary Figure 7

**Figure S7 Coexpression of TACI and CD138/BAFF-R**

Co-expression of TACI and CD138 (A) and of TACI and BAFF-R on splenic leukocytes, stimulated with CD40L and IL-10 is displayed.

Blots are gated for proliferating viable single cells.

## **5 unveröffentlichte Daten**

Im Folgenden sind die bisher unveröffentlichten aus den Untersuchungen der Harderschen Drüse in Form eines Manuskripts dargestellt.

# Characterization of HALT-derived plasma cells in chicken

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## Abstract

While the protective effect of most vaccines in chickens is primarily mediated by antibodies, the understanding of plasma cells (PC) in this species remains limited and PC-specific markers have been lacking. Additionally, given the fundamental differences in B cell development between birds and mammals, the potential to extrapolate mammalian data to chickens is highly restricted.

The Harderian gland (HG), a lacrimal gland in the eye orbit, had been described as leukocyte rich mucosa associated tissue, in which histologically many cells with PC-like morphology could be detected. Therefore, we have established an isolation protocol for HG leukocytes and by flow cytometry identified large CD45<sup>medium</sup> cells. Transcription factor profiling (Pax5<sup>neg</sup>, Blimp-1<sup>high</sup>) and microscopic examination of sort purified cells confirmed the plasma cell identity of this population.

These HG PCs had lost expression of BAFF-R and the chicken B cell marker chB6 but were all BCR positive. Immunoglobulin isotyping revealed a predominant class switch towards IgA, but also a subset of IgY<sup>pos</sup> cells. The PCs are homogenously positive for the (chicken B cell) activation marker CD57, which is in line with cell cycle analysis demonstrating that approximately 10% of the cells were dividing. High CXCR4 and switch from BAFF-R to low TACI expression of HG-PCs suggests the existence of survival niches like those known for mammalian PCs.

This study successfully isolated chicken plasma cells, confirmed their identity and characterized their phenotypes as CD45<sup>med</sup>/sIg<sup>+</sup>/CD57<sup>+</sup>/CXCR4<sup>high</sup> large cells. It revealed both conserved and species-specific aspects of mucosal plasma cell biology and establishes a valuable source for future functional studies.

# 1 Introduction

The mucosal immune system is a critical component of the body's defense mechanisms, protecting against pathogens that enter through mucosal surfaces such as the respiratory, gastrointestinal, and urogenital tracts (1). In chickens, plasma cells within mucosal tissues play a vital role in producing immunoglobulins (Ig) that contribute to mucosal immunity (2). These immunoglobulins are essential for neutralizing pathogens and preventing infections at mucosal surfaces. Despite their importance, chicken plasma cells have traditionally been identified microscopically due to the lack of specific surface markers (3).

To understand plasma cell development and function, insight into B-cell biology is required, shaped by the unique architecture of the chicken immune system.

Plasma cells, which represent terminally differentiated B cells, arise from a developmental process that begins with B-cell generation in the bursa of Fabricius, a unique lymphoid organ located near the cloaca (4).

During late embryonic development, a wave of prebursal stem cells committed to the B-cell lineage colonizes the bursa, where B cells proliferate and undergo somatic gene conversion to generate a diverse repertoire of antibodies (5). Once mature, B cells migrate from the bursa to peripheral lymphoid organs where they can encounter antigens.

Upon antigen exposure, B cells can form germinal centers (GCs) but in contrast to mammals where they form within splenic B-cell follicles, they form in arterial branchings within the T-cell zones (6). Although the GCs of the chicken seem to be more homogenous than the GCs of mammals with their clear separation between dark and light zone (7) there seems to be a spatial compartmentalization in the chicken (8).

Despite these differences, the function of chicken GCs is likely comparable to mammals, encompassing B-cell proliferation, affinity maturation, and immunoglobulin class switching.

One of the key mucosal tissues in chickens containing a substantial number of GCs is the Harderian gland (HG). This exocrine tubuloacinar gland of ectodermal origin is located within the orbit of the eye. Its primary function is to lubricate and maintain the nictitating membrane, but it also has significant immune functions. Structurally, the gland can be divided into a head and a body part based on differing lymphoid organization and surface epithelium (9). Histological studies have shown that the head region's lymphoepithelial tissue contains clusters of dendritic cells, macrophages, lymphocytes, and GCs, while the larger body part (9) contains numerous  $\text{chB6}^+$  B cells and plasma cells that stain positive for the EIV-E12 antibody (10). The Harderian gland's immune functions extend beyond immunoglobulin production, playing a role in the development and maintenance of mucosal immunity. The presence of lymphoid infiltration in germ-free chickens and the concentration of heterophils in the gland of chicken embryos suggest an antigen-independent or even an in-ovo stimulus for the induction of these structures (11). This indicates that the Harderian gland may have a role in the early development of the immune system in chickens.

Despite its critical role in producing immunoglobulins for the eye and upper respiratory tract and housing a substantial population of plasma cells, the Harderian gland remains relatively underexplored compared to other avian immune organs. Currently, plasma cell mechanisms cannot be precisely studied due to the lack of specific markers in chickens (3, 12).

Given the current situation, where Avian Influenza viruses continue to pose significant risks to both animal and human health, it is imperative to study the exact mechanisms by which these vaccines elicit immune responses, particularly in antibody-producing PCs (13-15). This

knowledge will enable the development of more effective vaccines that can be rapidly deployed to mitigate potential outbreaks.

Intraocular vaccines play a role not only in vaccination against avian influenza (16) but also but also in other viral diseases of chickens, like Newcastle virus. The Harderian gland, as a local immune organ, represents an important area of research in avian biology.

This paper will focus on the anatomical, histological, and immunological aspects of plasma cells within the Harderian gland, highlighting potential similarities in immune mechanisms between chickens and mammals, and instances of convergent evolution. Additionally, the study will develop a marker-based isolation protocol for characterizing plasma cells within the Harderian gland.

## 2 Results

### 2.1 Leukocyte Distribution in the Harderian gland

The Harderian Gland was isolated from a three-month-old chicken and prepared for histological examination. Consecutive sections of the plasma cell-rich head region (Figure 1A) were taken for immunohistological examination. Stainings revealed lymphoid tissue beneath the secretory duct epithelium composed of CD45<sup>+</sup> cells (Figure 1B). Within this tissue, chB6<sup>+</sup> and BAFF-R<sup>+</sup> B were primarily located just beneath the epithelium (Figure 1D, F). IgM, IgA, and IgY expressing plasma cells are diffusely distributed throughout the lymphoid tissue (Figure 1F-I). CD4<sup>+</sup> T helper cells are also broadly distributed but form distinct areas of higher cell density, suggesting localized aggregation (Figure 1J). Macrophages, identified by TIM4 expression, were uniformly dispersed throughout the lymphoid tissue (Figure 1K).

### 2.2 Identification of distinct leukocyte subpopulations by flow cytometry

Histology did not allow discrimination between cell sizes, so further analysis was carried out by flow cytometry using the CD45 pan-leukocyte marker.

The representation of CD45<sup>+</sup> cells versus cell-size (Figure 2A) revealed three distinct populations that can be described as small CD45<sup>medium</sup> cells, small CD45<sup>high</sup> cells and large CD45<sup>medium/high</sup> cells.

Staining for B (chB6) and T cell (CD3) lineage markers showed that 90% of the small CD45<sup>medium</sup> cells were surface immunoglobulin (slg) positive and 76% were chB6<sup>+</sup>, classifying them as B cells, with 6% CD3<sup>+</sup> T cells in this population (Figure 2B, E). Conversely, 84% of the small CD45<sup>high</sup> cells were CD3<sup>+</sup>, identifying them as T cells (Figure 2C, F). Detailed subtyping of the T lymphocyte subpopulation is in **Supplementary Figure 2**.

In contrast, the large cell population had low percentages of chB6<sup>+</sup> (2%) and CD3<sup>+</sup> (1%) cells (Figure 2D, G). However, 92% of these cells expressed slg, a characteristic of in-vitro generated chicken plasmablasts/-cells (17) and consistent with a plasma cell phenotype in mammalian literature (18, 19). This population also contained about 6% MRC1L-C<sup>+</sup> macrophages (Figure 2D, G).



## 2.3 Flow cytometric profile of putative HG plasma cells

To investigate whether the flow cytometric marker profile of ex-vivo plasma cells aligns with in-vitro generated plasma cells (17), additional markers were compared between chB6<sup>+</sup> B cells and putative plasma cells, defined as large CD45<sup>+</sup>/MRC1L-C<sup>-</sup> cells (**Figure 3A**).

As chB6 expression was completely reduced (**Figure 1D**) we examined the BAFF receptor, which showed a similar pattern. TACI, another receptor for BAFF expressed on both plasma and memory B in mammals (20-22), showed transient expression in in-vitro generated PCs (17) but, in contrast to B cells, was expressed, partly low, on all putative PCs isolated from the HG (**Figure 3A**). chCD138, which showed transient expression in *in-vitro* data (17), was present in only very few PCs and not at all on B cells. Compared to B cells, there was a clear reduction in MHCII expression in the large cell population.

CD57, a marker of B cell activation and potentially staining chPCs (17, 23) stained almost all putative PCs isolated from the HG (**Figure 3A**). This underscores the importance of including it in future PC-specific flow cytometric panels. Conversely, CD25, another activation marker, was present in nearly all B cells but only in a few large cells.

In mice, changes in responsiveness to chemokine receptor ligands, especially CXCL12 and CXCL13 (24, 25) prompted investigation of CXCR4 and CXCR5 expression in HG cells. CXCR4 was positive in most cells of both populations, with stronger staining in putative plasma cells. CXCR5 showed a contrasting pattern: all B cells stained strongly positive, while putative plasma cells had distinct positive and negative populations.

Initial stainings in CD45<sup>+</sup> cells for slg revealed a 10% discrepancy when individual Ig-isotypes were compared to Ig light chain-expressing cells (**Supplementary Figure 3**). This discrepancy was not observed with CD57 expression (**Figure 3B**; **Gating: Supplementary Figure 4A**). This approach revealed more IgY-expressing cells, suggesting steric hindrance between CD45 and IgY antibodies during surface staining. This hypothesis was supported by the absence of the gap when cells were permeabilized after CD45 staining and before Ig staining (**Figure 3C**).

## 2.4 Microscopic phenotype and cell cycle analysis

As the large cells, except for CXCR5, exhibited a uniform staining pattern for all markers analyzed by flow cytometry indicating a plasma cell-like phenotype, we investigated their microscopic appearance. This investigation revealed cells with a diameter of 6-10  $\mu\text{m}$ , a decentralized nucleus, and a cartwheel structure (**Figure 4**). Cell-cycle analysis showed a heterogeneous population with 10% of large cells in S-phase and 1.6% in G2/M phase, indicating ongoing cell division. The B cell population, on the other hand, was homogeneous with 99% of cells in G1 phase (**Figure 5**; **Gating: Supplementary Figure 4B**).

## 2.5 Transcriptomic differences between leukocyte subpopulations

Flow cytometric data (**Figure 2B-D, 3**) and microscopic analysis of sort-purified large CD45<sup>+</sup> subpopulations revealed them to be B- and T lymphocytes and cells with an expected plasma cell phenotype (**Figure 4**).

To investigate whether transcription factors are conserved between mammals and chickens, alongside the downregulation of B cell lineage markers and surface expression of different BCRs, we analyzed their profiles focusing on B- and plasma cell-specific factors and immunoglobulins by qRT-PCR. CD45<sup>+</sup> subpopulations were sort-purified, excluding monocytes/macrophages via MRC1L-C<sup>+</sup> staining.

The B cell-specific transcription factor *PAX5* was exclusively detected in the B cell population (**Figure 6A**). In contrast, *BLIMP1*, associated with plasma cells and an antagonist of *PAX5*, was predominantly expressed in plasma cells, with minimal levels in B cells (**Figure 6B**).

Regarding immunoglobulins, we analyzed  $\mu$ S (soluble form of the IgM heavy chain) and IgA. Both were present in B cells and plasma cells. However,  $\mu$ S expression was four times higher in plasma cells compared to B cells, while IgA expression was elevated by a factor of two in plasma cells relative to B cells (**Figure 6 C and D**).

### 3 Discussion

To optimize vaccine design in chicken, detailed knowledge of intracellular processes within B cells and specially the antibody-producing plasma cells is essential. Unfortunately, due to the lack of specific markers isolation of those cells in chicken has not been possible so far. Therefore, other strategies must be applied to isolate these specific cells.

Within leukocytes isolated of the chickens Harderian gland, by flow cytometry, we were able to detect cells that were substantially larger than classical B- and T lymphocytes and did express neither BAFF-R nor chB6 as B cell lineage markers, whilst strongly staining positive for surface BCR. The loss of chB6 in chicken PCs has already been described in histology (26) and was also seen in *in-vitro* data (17).

In terms of surface BCR expression we were able to detect all three isotypes of the chicken, IgM, IgA and IgY. This is in parts similar to mammalian data, where the expression of surface IgM and IgA has been described in plasma cells (18, 19) and contradicting the long time dogma that PCs do not express a surface BCR (27-29). However, we were able to find surface-bound IgY, the chicken analog for IgG, whereas IgG in human PCs seems to be intracellular only (30).

Interestingly, the observed isotype frequency distribution ( $\text{IgM} \geq \text{IgA} > \text{IgY}$ ) does not correspond to the immunoglobulin concentrations reported in tear fluid, where IgY is found at 2.3 mg/ml, IgM at 0.8 mg/ml, and IgA at 0.2 mg/ml (31). In contrast, Baba et al.(32) reported that in HG cell culture supernatants, IgA is the predominant isotype, followed by IgY, with only trace amounts of IgM. Based on these findings, the authors concluded that IgY and IgM in tear fluid must originate from sources other than the Harderian gland. While the observed isotype hierarchy in their study aligns with the sequential order of immunoglobulins in human tear fluid (33, 34), it is important to consider that no cytokines or other factors were added to the culture system. In mammalian plasma cell cultures, the addition of such factors has been shown to be critical for plasma cell survival (35, 36). Preliminary data from our laboratory (data not shown) suggest that this may also apply to HG plasma cells, as they did not survive beyond four days. Consequently, a decline in isotype-specific plasma cells may have influenced these results, underscoring the need for their reassessment.

Recently published potential plasma cell markers, chTACI and chCD138, have been shown to exhibit only transient expression *in-vitro* (17). However, in HG plasma cells, the entire population shifts toward TACI positivity, suggesting that all cells express chTACI, albeit some at low levels. The observed switch from BAFF-R to TACI as the primary BAFF receptor in chicken plasma cells is consistent with findings in humans (20, 37, 38). In contrast, only a small subset of HG plasma cells was positive for chCD138. Given the limited number of cells in active phases of the cell cycle, this supports the hypothesis that CD138 expression in chickens is more indicative of a specific activation state rather than serving as a constitutive marker of terminally differentiated cells.

Consistent with our *in vitro* findings (17) and previously published data (23), CD57 proved to be a reliable marker for plasma cells. However, since CD57 is not exclusively expressed on plasma cells, additional factors such as other markers or cell size should be considered in combination with CD57 for accurate identification.

The analysis and comparison of the chemokine receptor CXCR4 in plasma cells revealed expression patterns like those observed in humans (24, 25). Specifically, CXCR4 was

upregulated in plasma cells, which in humans—together with its binding partner CXCL12, produced by stromal cells in the bone marrow—is essential for the homing of long-lived plasma cells (LLPCs) into bone marrow niches. The present findings suggest that the HG may serve as a potential plasma cell survival niche, a hypothesis that is also supported by the high expression of the chemokine ligand CXCL12 in the gland that was detected by qRT-PCR (data not shown). However, the longevity of plasma cells in the HG, as well as chicken plasma cells in general, remains unknown.

The expression profile of chemokine receptor CXCR5 on the other hand differed from humans where it is known to be generally downregulated (24). Within the HG plasma cells we found two distinct populations (CXCR5<sup>+/+</sup>). This prompts the following question: to what extent do the two populations differ in terms of functionality?

The comparison of RT-qPCR results between B cells and plasma cells yielded expected findings, with significantly higher immunoglobulin expression levels in plasma cells. Even more pronounced were the differences in cell type-specific transcription factors, *PAX5* and *BLIMP1*. The downregulation of *Pax5*, accompanied by the simultaneous upregulation of *BLIMP1* in plasma cells, is consistent not only with in vitro data from chickens (17) but also with findings in humans (39-41), highlighting another conserved mechanism in the differentiation process from B cells to plasma cells.

In conclusion, this study not only enabled the isolation of plasma cells from the chicken HG using a combination of cell surface markers (large CD45<sup>+</sup>/CD57<sup>+</sup>/BCR<sup>+</sup>/lineage marker<sup>+</sup> cells) but also provided evidence for conserved mechanisms in plasma cell differentiation. These include the up- and downregulation of specific cell surface markers, alterations in chemokine receptor profiles, and shifts in transcription factor expression. This deeper understanding allows for a more targeted approach to studying plasma cells and their underlying mechanisms in the future, particularly in the context of antibody responses and vaccine development.

## 4 Data limitations and perspectives

Within the small CD45<sup>medium</sup> cells, we found around 14% expressing surface immunoglobulin but not chB6. As this population partly consisted of class-switched cells, they could represent memory cells. However, virtually nothing is known about this cell type in chickens. Evidence for their existence comes from their involvement in antibody recall responses following prime-boost vaccinations (42). A detailed characterization of this population was beyond the scope of this study but will be addressed in future investigations.

Having identified several conserved mechanisms in plasma cell development between human and chicken plasma cells, our next step will be to adapt plasma cell culture approaches from mammalian systems to chickens. This will enable us to investigate the antibody production of Harderian gland-derived plasma cells in greater detail.

## 5 Material & Methods

### 5.1 Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature, incubated in 15% sucrose overnight at 4°C, then infiltrated with phosphate buffered saline (PBS) containing 7.5% gelatin and 15% sucrose for 2 hours at 37°C. The impregnated tissue samples were embedded in the same 7.5% gelatin medium then rapidly frozen at -50°C in 2-methylbutane (Sigma, 78-78-4). 12 µm cryosections were collected on poly-L-lysine (Sigma, P8920) coated slides and incubated with primary antibodies (Table 1) for 1 hour at room temperature, followed by biotinylated goat anti-mouse IgG (Vector Laboratories, BA-9200) secondary antibody and avidin-biotinylated peroxidase complex (ABC; Vectastain Elite ABC kit, Vector Laboratories, PK-6100). Endogenous peroxidase activity was quenched by 3% hydrogen-peroxide (Sigma, H1009) in PBS for 10 minutes. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (Sigma, C8890).

For double immunofluorescence, sections were incubated with primary antibodies (Table 1) for 1 hour at room temperature followed by Alexa-conjugated fluorescent secondary antibodies (Table 2).

Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, California) and sections were covered by aqueous Poly/Mount (Polyscience Inc. Warrington, PA, 18606).

Section images were recorded using a Nikon Eclipse E800 fluorescence microscope and Zeiss LSM 710 confocal microscope. Image processing was performed using CellSens, ZEISS ZEN Imaging proprietary software and ImageJ. Images were compiled using Adobe Photoshop 7.0.

### 5.2 Animals

Fertilized eggs of M11 white layer chicken (B2/B2 haplotyp) were provided by Prof. Dr. Steffen Weigend (FLI Mariensee) and incubated and hatched at the Faculty of Veterinary Medicine, LMU Munich. Animals did not receive vaccinations and were housed in groups under conventional conditions and received both food and water ad-libitum.

Experiments with M11 birds were performed at the age of 12-24 weeks.

### 5.3 Cell preparation

The animals were euthanized through exsanguination following anesthesia. The glands were aseptically dissected and placed in transport media (RPMI 1640, Thermo Fisher Scientific, Waltham, USA) supplemented with 1% Penicillin-Streptomycin (Merck KGaA, Darmstadt, Germany) at room temperature (RT) until further processing.

Single cell suspensions were obtained by mechanical dissociation of the tissue through a stainless-steel. Subsequently single cell suspensions were filtrated through one-time folded lens paper (THORLABS GmbH, Bergkirchen, Germany) and leukocyte separation was accomplished by density gradient centrifugation with Lymphocyte Separation Medium (Capricorn Scientific GmbH, Ebsdorfergrund, Germany).

### 5.4 Flow cytometry and cell sorting

Staining of cells for flow cytometric analysis was performed according to standard procedures. Briefly,  $10^{5-6}$  cells were transferred to a 96-well plate and incubated with Fixable Viability Dye



eFluor™ 780 dilution (Thermo Fisher Scientific, Waltham, USA). Subsequently, cells were stained with 50µl of monoclonal antibody (mAb) mixture. mAbs were either directly conjugated or detected by fluorochrome conjugated secondary antibodies (**Supplementary Table 1 and 2**). If necessary, when the unconjugated primary antibodies and the direct conjugates were of the same isotype, a blocking step with normal mouse serum (Jackson ImmunoResearch, West Grove, USA) was interposed before applying the directly conjugated antibody.

All incubation steps were carried out for 20 minutes in the dark on ice. In between the incubation steps, cells were washed with 200µl of staining buffer (PBS pH 7.2 (Thermo Fisher Scientific, Waltham, USA), 1% bovine serum albumin and 0.1% NaN<sub>3</sub> (ITW Reagents, Castellar del Vallès, Spain)).

For intracellular staining, cells were first subjected to surface staining, followed by fixation with eBioscience™ IC Fixation Buffer (Thermo Fisher Scientific, Waltham, USA) for 20 minutes on ice. The cells were then washed three times, each for one minute, using eBioscience™ Permeabilization Buffer (Thermo Fisher Scientific, Waltham, USA). Intracellular staining antibodies were diluted in the same permeabilization buffer, and the cells were incubated with these antibodies for 30 minutes. Following this, the cells were washed three times for one minute before proceeding with staining using fluorochrome-conjugated secondary antibodies.

The anti-chicken L chain mAb (**Supplementary Table 1**) was conjugated to CF405M using the Mix-n-Stain™ CF™ 405M Antibody Labeling Kit (Merck KGaA, Darmstadt, Germany).

The anti-chicken CD45 mAb was conjugated to FITC, APC or PerCp using the corresponding LYNX Rapid conjugation kit (Bio-Rad Laboratories Ltd., Watford, UK).

Flow cytometry was performed with a FACSCanto II (Becton Dickinson, Heidelberg, Germany) and data were analyzed using FACSDiva (Becton Dickinson, Heidelberg, Germany) and FlowJo (FlowJo LLC, Oregon, USA) software, version 10.10.0.

Sort purification of cells was performed on a FACSARIA Illu (Becton Dickinson, Heidelberg, Germany) with FACS DIVA software. Cells that were subjected for later qRT-PCR analysis were sorted directly into the lysis buffer of the ReliaPrep™ RNA Miniprep Systems kit (Promega, Madison, USA).

## 5.5 Cytospin

Sort purified cells were washed resuspended in 200µl PBS.

The resuspended cells were added to cytofunnels (EpreDia™ Shandon™ Single Cytofunnel™; Fisher Scientific GmbH, Schwerte, Germany) and centrifuged on SuperFrost® Plus glass slides (Thermo Fisher Scientific, Waltham, USA) using a Cytospin 4 cytocentrifuge (Thermo Fisher Scientific, Waltham, USA) for 10 minutes / 500g.

Afterwards cells were fixed with methanol and stained with Giemsa staining solution (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

## 5.6 Cell cycle analysis

After staining for CD45 and MRC1L-C cells were fixed and permeabilized in cold 70% ethanol for 2 hours on ice. Then cells were centrifuged and washed with PBS. The cell pellet was then resuspended in 1ml staining buffer (500µl propidium iodide (PI) solution (100µg/ml) (Merck KGaA, Darmstadt, Germany) + 490µl PBS pH 7.2 (Thermo Fisher Scientific, Waltham, USA) + 10µl RNaseA (Thermo Fisher Scientific, Waltham, USA)) and the cells were incubated for 15 minutes at 37°C in a water bath.

PI linear fluorescence was measured immediately afterwards with a FACSCanto II (Becton Dickinson, Heidelberg, Germany) and the data was analyzed using FlowJo (FlowJo LLC, Oregon, USA) software, version 10.10.0.

## 5.7 qRT-PCR

For qRT-PCR, RNA of sort purified CD45<sup>+</sup> subpopulations and CD45<sup>-</sup> viable single cells, was prepared using the ReliaPrep™ RNA Miniprep Systems kit (Promega, Madison, USA) following the manufacturer's instructions.

The RNA was quantified on a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) as well as on a Quantus™ Fluorometer (Promega, Madison, USA) and RNA quality was determined on a 2100 Bioanalyzer (RIN > 9) (Agilent Technologies, Santa Clara, CA, USA). cDNA was prepared using the GoScript™ Reverse Transcriptase kit (Promega, Madison, USA) according to manufacturer's instruction and analyzed for the relative abundance of *RPL13*, *μS*, *IgA*, *PAX5*, and *BLIMP1* using primers from Eurofins Genomics Germany GmbH (Ebersberg, Germany) specified in **Supplementary Table 3** and the GoTaq® qPCR MasterMix (Promega, Madison, USA).

Quantitative RT-PCR was performed using a 7300 Real-Time PCR System® (Applied Biosystems, Warrington, UK) with SYBR-green.

Relative expression fold change for the respective genes was assessed using normalized cycle threshold values ( $\Delta$ CT). Normalization was carried out against the expression in sort purified CD45<sup>-</sup> viable single cells.

## 6 Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author

## 7 Conflict of interest

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

## 8 Ethics statement

Organ collection was done in accordance with federal, local and European legislation as well as institutional requirements.

## 9 Authors contribution

DvLR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing; IN: Formal analysis, Investigation, Methodology, Validation, Visualization; ES: Formal analysis, Investigation, Methodology, Validation, Visualization; MK: Formal analysis, Investigation, Methodology; NN: Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing

SH: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing

All authors contributed to manuscript revision, read and approved the submitted version.

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## 11 References

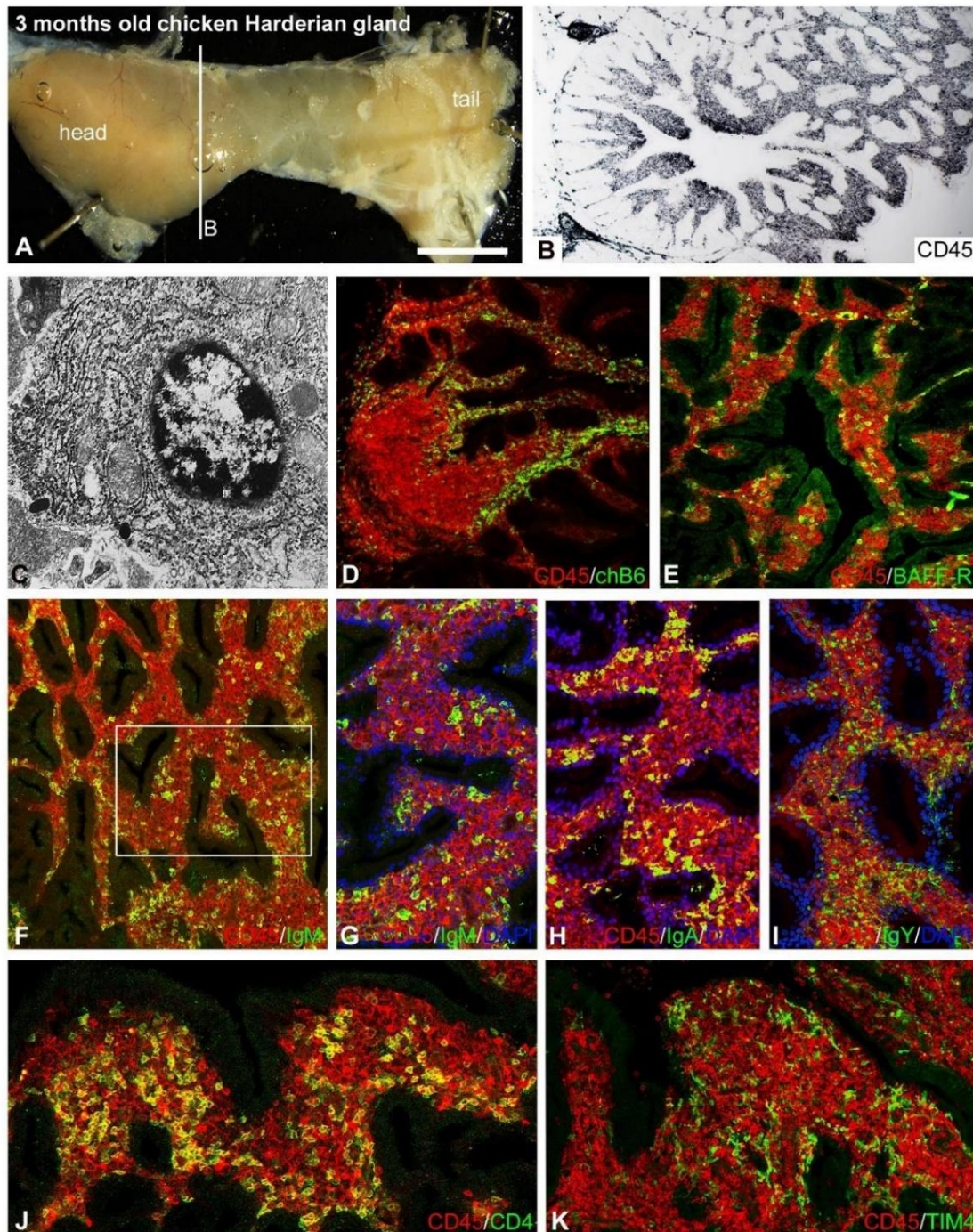
1. Nochi T, Jansen CA, Toyomizu M, van Eden W. The Well-Developed Mucosal Immune Systems of Birds and Mammals Allow for Similar Approaches of Mucosal Vaccination in Both Types of Animals. *Front Nutr.* 2018;5:60.
2. Guralaska SV, Kot TF, Dyshliuk NV, Zaika SS, Khomenko ZV. Immune Response of the Harderian Gland in Chickens to Infectious Bronchitis Coronavirus. *Agr Sci Pract.* 2021;8(1):58-66.
3. Ratcliffe MJ, Härtle S. B cells, the bursa of Fabricius, and the generation of antibody repertoires. In: Kaspers B, Schat KA, Göbel TW, Vervelde L, editors. *Avian Immunology*. 3 ed. United Kingdom: Academic Press, Elsevier Ltd.; 2022. p. 71-99.
4. Davison F. The Importance of the Avian Immune System and Its Unique Features. In: Kaspers B, Schat KA, Göbel TW, Vervelde L, editors. *Avian Immunology*. 3 ed. United Kingdom: Academic Press, Elsevier Ltd.; 2022. p. 1-11.
5. Ratcliffe MJH. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. *Developmental and Comparative Immunology.* 2006;30(1-2):101-18.
6. Romppanen T. A morphometrical method for analyzing germinal centers in the chicken spleen. *Acta Pathol Microbiol Scand C.* 1981;89(4):263-8.
7. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity.* 2007;27(2):190-202.
8. Yasuda M, Taura Y, Yokomizo Y, Ekino S. A comparative study of germinal center: fowls and mammals. *Comp Immunol Microbiol Infect Dis.* 1998;21(3):179-89.
9. Olah I, Kupper A, Kittner Z. The lymphoid substance of the chicken's harderian gland is organized in two histologically distinct compartments. *Microsc Res Tech.* 1996;34(2):166-76.
10. Pharr T, Olah I, Bricker J, Olson WC, Ewert D, Marsh J, et al. Characterization of a novel monoclonal antibody, EIV-E12, raised against enriched splenic ellipsoid-associated cells. *Hybridoma.* 1995;14(1):51-7.
11. Bang BG, Bang FB. Localized lymphoid tissues and plasma cells in paraocular and paranasal organ systems in chickens. *Am J Pathol.* 1968;53(5):735-51.
12. Hartle S, Sutton K, Vervelde L, Dalgaard TS. Delineation of chicken immune markers in the era of omics and multicolor flow cytometry. *Front Vet Sci.* 2024;11:1385400.
13. Pascua PN, Choi YK. Zoonotic infections with avian influenza A viruses and vaccine preparedness: a game of "mix and match". *Clin Exp Vaccine Res.* 2014;3(2):140-8.
14. Everest H, Billington E, Daines R, Burman A, Iqbal M. The Emergence and Zoonotic Transmission of H10Nx Avian Influenza Virus Infections. *mBio.* 2021;12(5):e0178521.
15. Kang M, Wang LF, Sun BW, Wan WB, Ji X, Baele G, et al. Zoonotic infections by avian influenza virus: changing global epidemiology, investigation, and control. *Lancet Infect Dis.* 2024;24(8):e522-e31.
16. Hikono H, Mase M, Matsuu A, Nakayama M, Saito T. Intraocular vaccination with an inactivated highly pathogenic avian influenza virus induces protective antibody responses in chickens. *Vet Immunol Immunopathol.* 2013;151(1-2):83-9.
17. von La Roche D, Schumacher M, Kohn M, Trapp J, Schusser B, Rautenschlein S, et al. Characterization of class-switched B cells in chickens. *Front Immunol.* 2024;15:1484288.
18. Pinto D, Montani E, Bolli M, Garavaglia G, Sallusto F, Lanzavecchia A, et al. A functional BCR in human IgA and IgM plasma cells. *Blood.* 2013;121(20):4110-4.

19. Blanc P, Moro-Sibilot L, Barthly L, Jagot F, This S, de Bernard S, et al. Mature IgM-expressing plasma cells sense antigen and develop competence for cytokine production upon antigenic challenge. *Nat Commun.* 2016;7:13600.
20. Garcia-Carmona Y, Cols M, Ting AT, Radigan L, Yuk FJ, Zhang L, et al. Differential induction of plasma cells by isoforms of human TACI. *Blood.* 2015;125(11):1749-58.
21. Sanz I, Wei C, Jenks SA, Cashman KS, Tipton C, Woodruff MC, et al. Challenges and Opportunities for Consistent Classification of Human B Cell and Plasma Cell Populations. *Front Immunol.* 2019;10:2458.
22. Delaloy C, Schuh W, Jack HM, Bonaud A, Espeli M. Single-cell resolution of plasma cell fate programming in health and disease. *Eur J Immunol.* 2022;52(1):10-23.
23. Mast J, Goddeeris BM. CD57, a marker for B-cell activation and splenic ellipsoid-associated reticular cells of the chicken. *Cell Tissue Res.* 1998;291(1):107-15.
24. Hargreaves DC, Hyman PL, Lu TT, Ngo VN, Bidgol A, Suzuki G, et al. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med.* 2001;194(1):45-56.
25. Cyster JG, Ansel KM, Ngo VN, Hargreaves DC, Lu TT. Traffic patterns of B cells and plasma cells. *Adv Exp Med Biol.* 2002;512:35-41.
26. Houssaint E, Diez E, Pink JR. Ontogeny and tissue distribution of the chicken Bu-1a antigen. *Immunology.* 1987;62(3):463-70.
27. Nossal GJ, Pike BL. Differentiation of B lymphocytes from stem cell precursors. *Adv Exp Med Biol.* 1973;29(0):11-8.
28. Tyler BM, Cowman AF, Gerondakis SD, Adams JM, Bernard O. mRNA for surface immunoglobulin gamma chains encodes a highly conserved transmembrane sequence and a 28-residue intracellular domain. *Proc Natl Acad Sci U S A.* 1982;79(6):2008-12.
29. Sitia R, Neuberger MS, Milstein C. Regulation of membrane IgM expression in secretory B cells: translational and post-translational events. *EMBO J.* 1987;6(13):3969-77.
30. Jourdan M, Caraux A, De Vos J, Fiol G, Larroque M, Cognot C, et al. An in vitro model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. *Blood.* 2009;114(25):5173-81.
31. Baba T, Masumoto K, Nishida S, Kajikawa T, Mitsui M. Harderian gland dependency of immunoglobulin A production in the lacrimal fluid of chicken. *Immunology.* 1988;65(1):67-71.
32. Baba T, Kawata T, Masumoto K, Kajikawa T. Role of the harderian gland in immunoglobulin A production in chicken lacrimal fluid. *Res Vet Sci.* 1990;49(1):20-4.
33. McClellan BH, Whitney CR, Newman LP, Allansmith MR. Immunoglobulins in tears. *Am J Ophthalmol.* 1973;76(1):89-101.
34. Donshik PC, Ballow M. Tear immunoglobulins in giant papillary conjunctivitis induced by contact lenses. *Am J Ophthalmol.* 1983;96(4):460-6.
35. Minges Wols HA, Underhill GH, Kansas GS, Witte PL. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *J Immunol.* 2002;169(8):4213-21.
36. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, et al. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol.* 2003;171(4):1684-90.
37. Smulski CR, Eibel H. BAFF and BAFF-Receptor in B Cell Selection and Survival. *Front Immunol.* 2018;9:2285.
38. Good KL, Avery DT, Tangye SG. Resting Human Memory B Cells Are Intrinsically Programmed for Enhanced Survival and Responsiveness to Diverse Stimuli Compared to Naive B Cells. *Journal of Immunology.* 2009;182(2):890-901.
39. Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity.* 2002;17(1):51-62.
40. Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. *Nat Rev Immunol.* 2005;5(3):230-42.

41. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity*. 2003;19(4):607-20.
42. Nagase F, Nakashima I, Kato N. Studies on the immune response in chickens. IV. Generation of hapten-specific memory and absence of increase in carrier-specific helper memory in antibody response to sheep red blood cell antigen and its hapten-conjugate. *Dev Comp Immunol*. 1983;7(1):127-37.

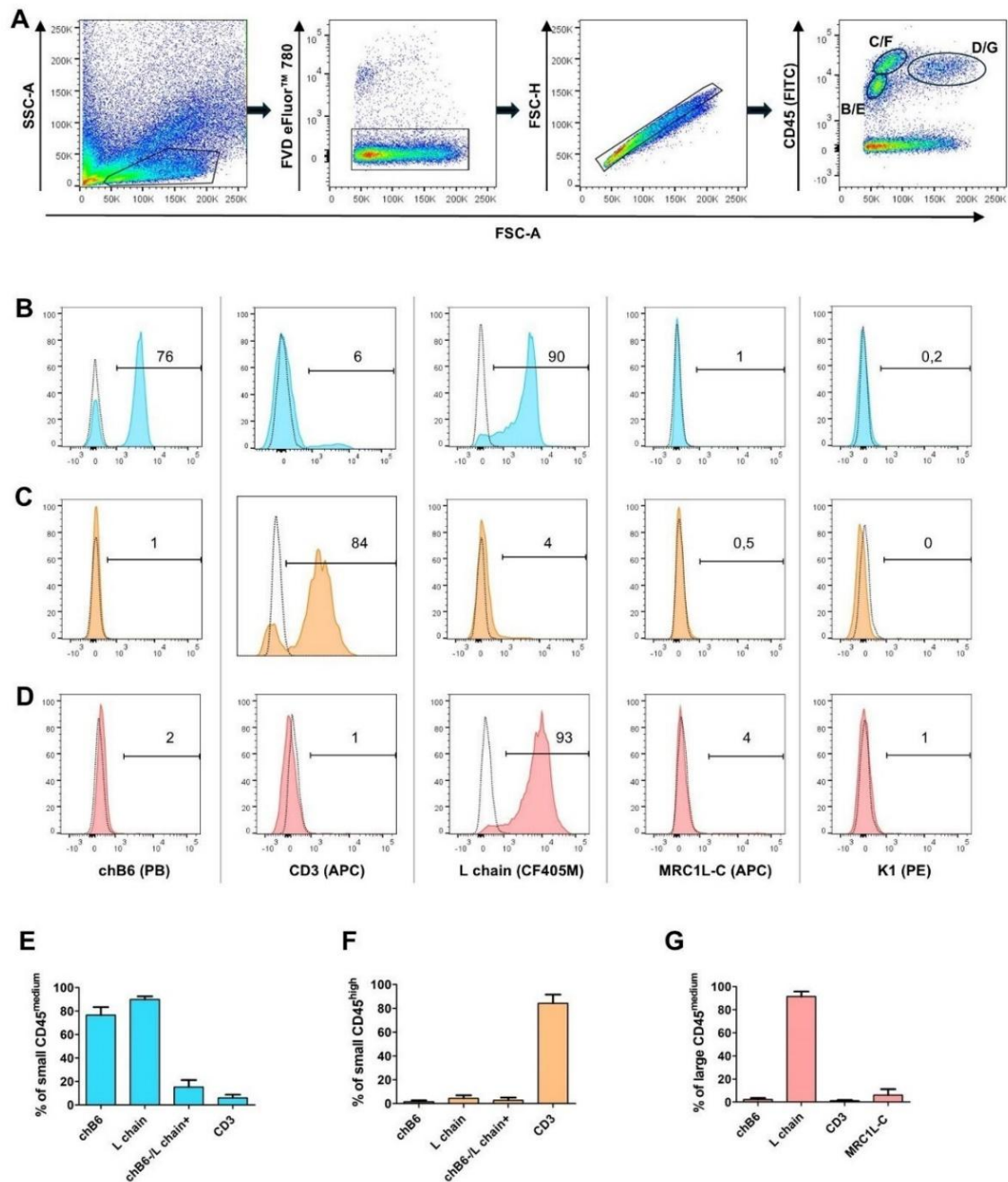


## 12 Figures



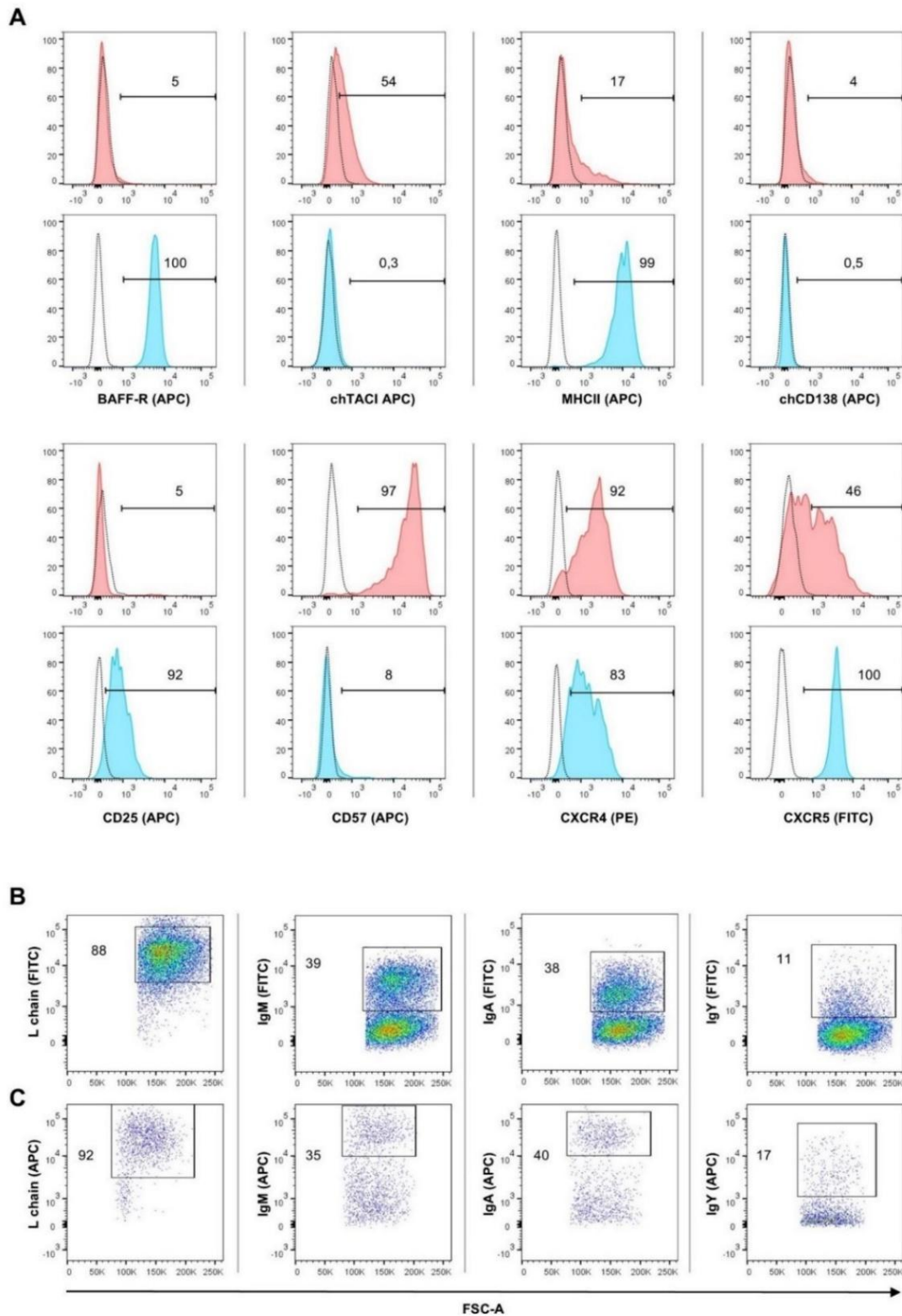
**Figure1 Leukocyte distribution in the Harderian gland.**

(A) Freshly dissected HG, the line indicating the orientation of histological sections. (B) Localization of CD45<sup>+</sup> leukocytes. (C) Visualization of a plasma cell nucleus and endoplasmic reticulum by electron microscopy. (D, E) Expression patterns of B cell markers chB6 and BAFF-R (F–I) immunoglobulins. (J) CD4<sup>+</sup> T helper cells and. (K) TIM4<sup>+</sup> macrophages. Scale bar indicates 2mm (A) 200  $\mu$ m (B), 6  $\mu$ m (C), 140  $\mu$ m (D), 100  $\mu$ m (E), 85  $\mu$ m (F), 50  $\mu$ m (G–I), 60  $\mu$ m (J) and 80  $\mu$ m (K).



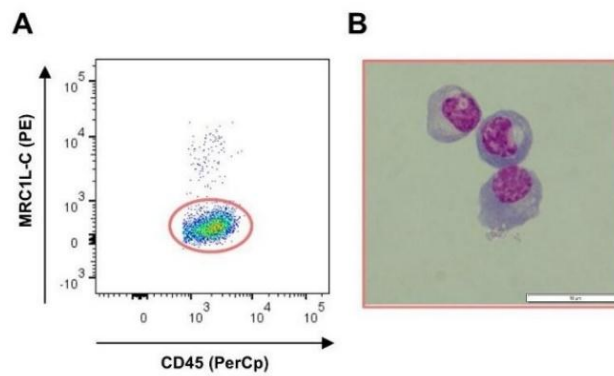
**Figure 2: Identification of characteristic CD45<sup>+</sup>/FSC based leukocyte subsets in isolated HG cells.**  
 (A) Gating strategy to identify different leukocyte subsets in HG preparations according to FSC and CD45 expression.  
 (C-D) Representative lineage marker expression within CD45<sup>+</sup> subsets indicated by the letters next to the gates in A.  
 (E-G) Cellular composition in gates indicated in A; mean  $\pm$  SD from nine independent experiments.





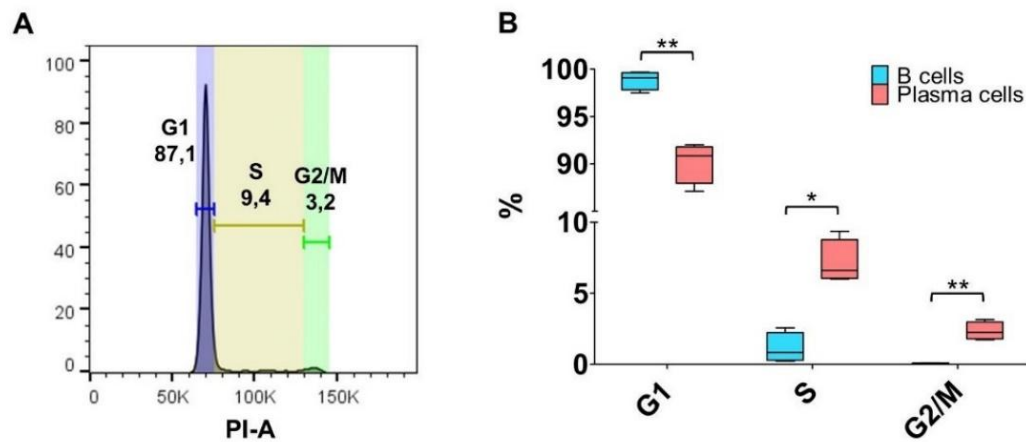
**Figure 3: Comparison of small and large sIg<sup>+</sup> cells**

(A) HG leukocytes were gated for viable, single small CD45<sup>medium</sup>/chB6<sup>+</sup> cells (blue) and large, CD45<sup>medium</sup>/MRC1L-C<sup>+</sup> cells (red) and analyzed by flow cytometry for expression of the indicated surface markers (dotted line indicate respective isotype controls). (B,C) Comparison of surface (B) and surface plus intracellular (C) Ig expression on large CD45<sup>+</sup>/CD57<sup>+</sup> cells. Data shown are representative for three independent experiments.



**Figure 4: Plasma cell morphology of large CD45<sup>med</sup>/MRC1L-C<sup>-</sup> cells**

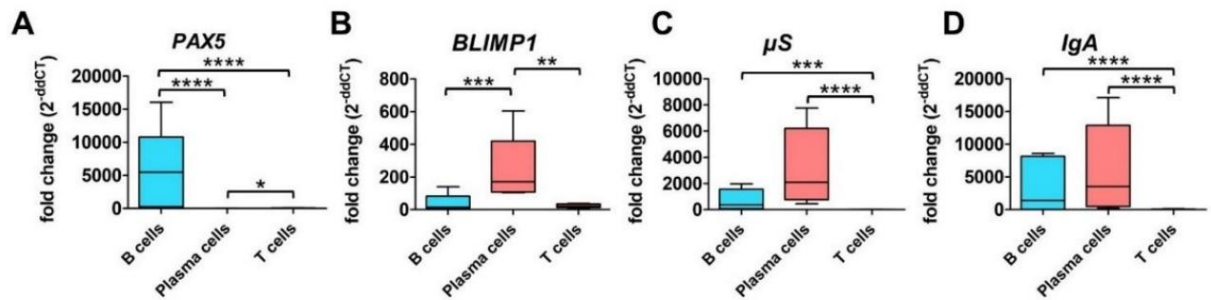
(A) Gating for putative plasma cells in HG leukocytes (plot shows viable, single large CD45<sup>med</sup> cells); (B) Giemsa stained Cytospin of sort purified CD45<sup>medium</sup>/MRC1L-C<sup>-</sup> cells. (scale bar = 10 μm).



**Figure 5 Cell division in the plasma cell gate**

(A) Representative histogram of PI staining for DNA content / cell cycle analysis of B cells and plasma cells using the FlowJo 10.10.0 cell cycle tool. (B) Mean  $\pm$  SD of cell cycle phases from four independent experiments. with \*:  $p \leq 0.05$  and \*\*:  $p \leq 0.01$ . HG leukocytes were gated for viable, single small CD45<sup>medium</sup> cells (b cells) and large, CD45<sup>medium</sup> /MRC1L-C cells (plasma cells).





**Figure 6: : Distinct transcription factor and Ig expression profile of HG plasma cells**

HG cells were sort-purified as B cells (small CD45<sup>medium</sup>) and plasma cells (large CD45<sup>medium</sup>/MRC1L-C-) and expression of the respective genes was determined by qRT-PCR and normalized against CD45<sup>negative</sup> cells. Statistics were calculated on  $\Delta\text{CT}$  values by 1way ANOVA with: \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$  and \*\*\*\*:  $p \leq 0.0001$ . Shown are mean  $\pm$  SD of five independent experiments with pooled tissue of four animals per experiment.

## 13 Supporting Information

**Supplementary Table 1**

Primary mAbs and directly conjugated mAbs.

| Antigen              | Clone | Species | Isotype | Fluorochrome | Source of antibody    |
|----------------------|-------|---------|---------|--------------|-----------------------|
| chB6                 | AV20  | Mouse   | IgG1    |              | Southern Biotech      |
| chB6                 | AV20  | Mouse   | IgG1    | Pacific blue | Southern Biotech      |
| chBAFF-receptor      | 2C4   | Mouse   | IgG1    |              | (1)                   |
| chMHCII              | 2G11  | Mouse   | IgG1    |              | Southern Biotech      |
| IgY                  | G1    | Mouse   | IgG1    |              | Southern Biotech      |
| chCXCR5              | 6A9   | Mouse   | IgG1    |              | (2)                   |
| TCR $\gamma\delta$   | TCR1  | Mouse   | IgG1    |              | Southern Biotech      |
| TCR $\alpha\beta$ -1 | TCR2  | Mouse   | IgG1    | FITC         | Southern Biotech      |
| TCR $\alpha\beta$ -2 | TCR3  | Mouse   | IgG1    | FITC         | Southern Biotech      |
| chCD4                | CT4   | Mouse   | IgG1    | FITC         | Southern Biotech      |
| chCD138              | 7G10  | Mouse   | IgG1    |              | (3)                   |
| MRC1L-C              | Kul01 | Mouse   | IgG1    |              | Southern Biotech      |
| chE-cadherin         | 7D6   | Mouse   | IgG1    |              | DSHB                  |
| chL chain            | 2G1   | Mouse   | IgG1    | CF405M       | BioRad                |
| chL chain            | 2G1   | Mouse   | IgG1    |              | BioRad                |
| IgA                  | K3    | Mouse   | IgG2a   |              | (4)                   |
| chCXCR4              | 9D9   | Mouse   | IgG2a   |              | (5)                   |
| chCD28               | 2-4   | Mouse   | IgG2a   |              | (6)                   |
| chCD45               | 16-6  | Mouse   | IgG2a   |              | (7)                   |
| chCD45               | 16-6  | Mouse   | IgG2a   | FITC         | (7)                   |
| chCD45               | 16-6  | Mouse   | IgG2a   | APC          | (7)                   |
| chCD45               | 16-6  | Mouse   | IgG2a   | PerCP        | (7)                   |
| unknown              | K1    | Mouse   | IgG2a   |              | (8)                   |
| IgM                  | M1    | Mouse   | IgG2b   |              | Southern Biotech      |
| IgA                  | A1    | Mouse   | IgG2b   |              | Southern Biotech      |
| chCD8                | 3-298 | Mouse   | IgG2b   |              | (9)                   |
| chTACI               | 1H4   | Mouse   | IgG2b   |              | (3)                   |
| chCD25               | 28-4  | Mouse   | IgG3    |              | (10)                  |
| Human CD57           | NK1   | Mouse   | IgM     | APC          | BD-Bioscience         |
| TIM4                 | JH9   | Mouse   | IgG1    |              | Roslin Institute (11) |

**Supplementary Table 2**

Fluorochrome conjugated secondary antibodies.

| Antigen      | Fluorochrome        | Source of antibody      |
|--------------|---------------------|-------------------------|
| Mouse-IgG1   | PE                  | Jackson Laboratories    |
| Mouse-IgG1   | APC                 | Jackson Laboratories    |
| Mouse-IgG1   | FITC                | Southern Biotech        |
| Mouse-IgG1   | Brilliant Violet421 | Jackson Laboratories    |
| Mouse-IgG1   | AF488               | ThermoFisher Scientific |
| Mouse-IgG2a  | FITC                | Southern Biotech        |
| Mouse-IgG2a  | Alexa Fluor 647     | Invitrogen              |
| Mouse-IgG2a  | AF594               | ThermoFisher Scientific |
| Mouse-IgG2b  | RPE                 | Southern Biotech        |
| Mouse-IgG2b  | APC                 | Southern Biotech        |
| Mouse-IgG2b  | AF488               | ThermoFisher Scientific |
| Mouse-IgG3   | FITC                | Southern Biotech        |
| Mouse-IgG3   | Alexa Fluor 647     | Southern Biotech        |
| Streptavidin | APC                 | Jackson Laboratories    |

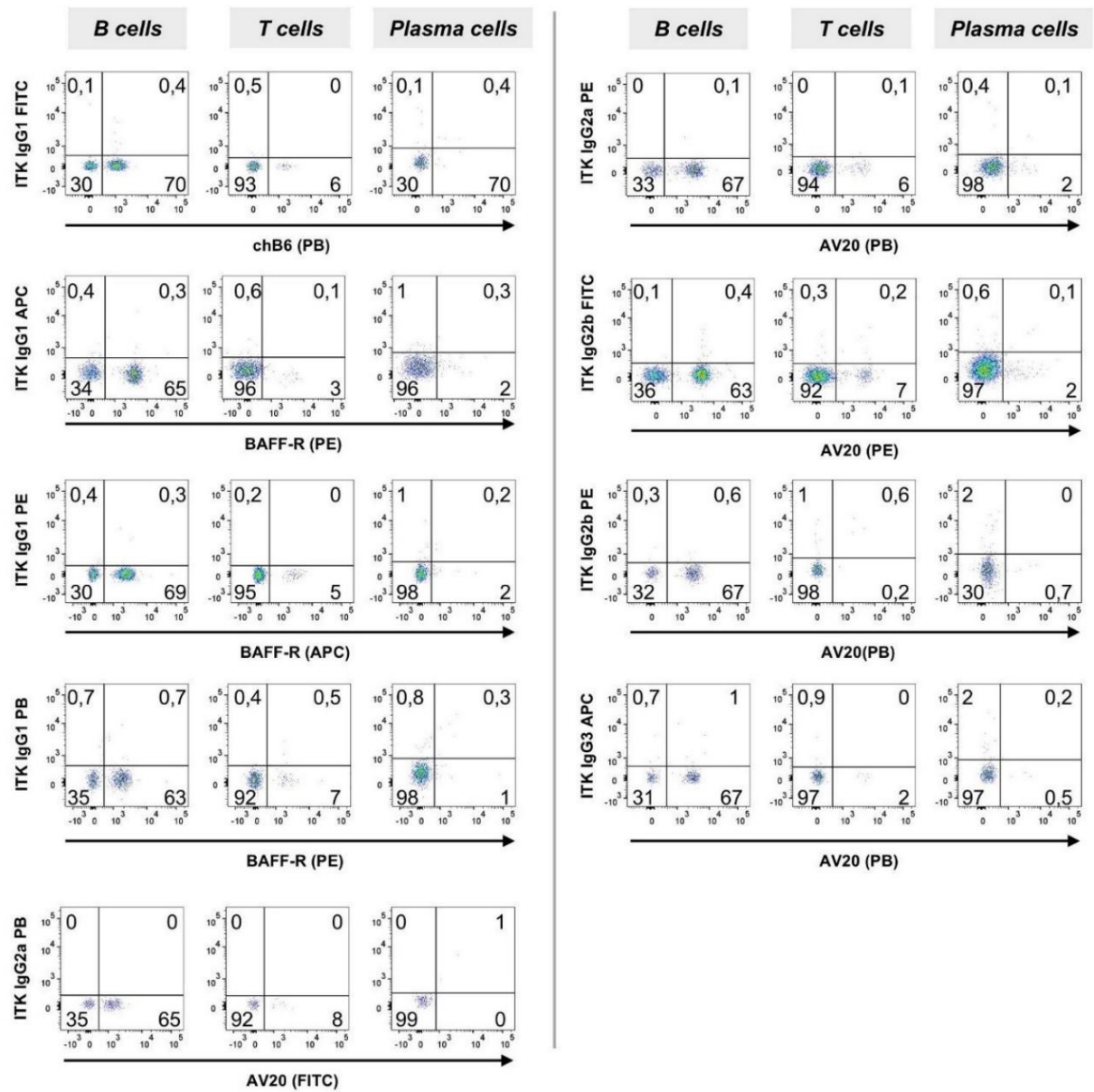
**Supplementary Table 3**

Forward and reverse primers used for qRT-PCR

| Gen           | Forward primer               | Reverse Primer                 |
|---------------|------------------------------|--------------------------------|
| <b>RPL 13</b> | 5'- GAGGTGCCCGACTGTCAGAT-3'  | 5'- ATCGTCCGAGCAAACCTTTTGT-3'  |
| <b>Pax 5</b>  | 5'- CCAGCAGCAGTTGGAAGTGTT-3' | 5'- CTCTGGTTTGATGGGCTCTGTT-3'  |
| <b>Blimp1</b> | 5'- GTGGTATTGCCGAGACTTTGC-3' | 5'-GGGTTTGTGTGAGGTTTCATCATT-3' |
| <b>μS</b>     | 5'- GGAGAACCCCGAAAATGAGT-3'  | 5'- GCCAACACCAAGGAGACATT-3'    |
| <b>IgA</b>    | 5'- CGCCCCTTCCGTCTACGT-3'    | 5'- CGAAATCGGTTGGTTTGTG-3'     |

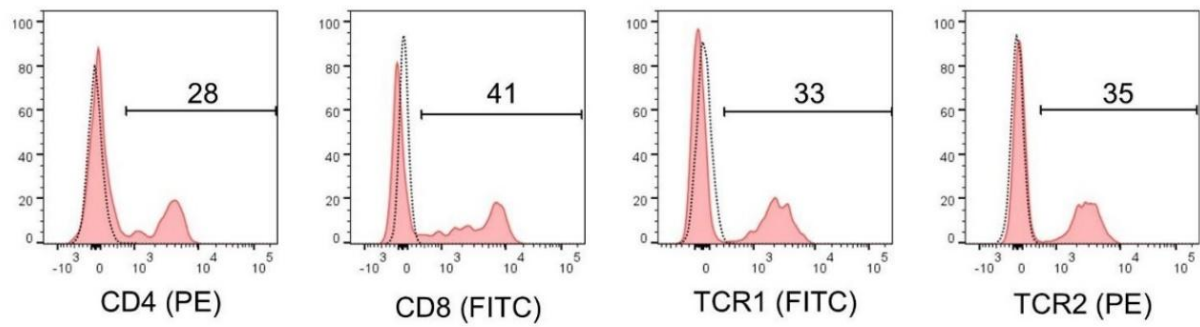
1. Jax E, Werner E, Muller I, Schaerer B, Kohn M, Olofsson J, et al. Evaluating Effects of AIV Infection Status on Ducks Using a Flow Cytometry-Based Differential Blood Count. *Microbiol Spectr.* 2023;11(4):e0435122.
2. Haertle S, Alzuheir I, Busalt F, Waters V, Kaiser P, Kaufer BB. Identification of the Receptor and Cellular Ortholog of the Marek's Disease Virus (MDV) CXCL Chemokine. *Front Microbiol.* 2017;8:2543.
3. von La Roche D, Schumacher M, Kohn M, Trapp J, Schusser B, Rautenschlein S, et al. Characterization of class-switched B cells in chickens. *Front Immunol.* 2024;15:1484288.
4. Erhard MH, Von Quistorp I, Schraner I, Jungling A, Kaspers B, Schmidt P, et al. Development of specific enzyme-linked immunosorbent antibody assay systems for the detection of chicken immunoglobulins G, M, and A using monoclonal antibodies. *Poult Sci.* 1992;71(2):302-10.
5. Escot S, Blavet C, Hartle S, Duband JL, Fournier-Thibault C. Misregulation of SDF1-CXCR4 signaling impairs early cardiac neural crest cell migration leading to conotruncal defects. *Circ Res.* 2013;113(5):505-16.
6. Arstila TP, Vainio O, Lassila O. Evolutionarily conserved function of CD28 in alpha beta T cell activation. *Scand J Immunol.* 1994;40(3):368-71.
7. Viertlboeck BC, Gobel TW. Chicken thrombocytes express the CD51/CD61 integrin. *Vet Immunol Immunopathol.* 2007;119(1-2):137-41.

8. Kaspers B, Lillehoj HS, Lillehoj EP. Chicken macrophages and thrombocytes share a common cell surface antigen defined by a monoclonal antibody. *Vet Immunol Immunopathol.* 1993;36(4):333-46.
9. Norup LR, Dalgaard TS, Pedersen AR, Juul-Madsen HR. Assessment of Newcastle disease-specific T cell proliferation in different inbred MHC chicken lines. *Scand J Immunol.* 2011;74(1):23-30.
10. Gobel TW, Kaspers B, Stangassinger M. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. *Int Immunol.* 2001;13(6):757-62.
11. Hu T, Wu Z, Bush SJ, Freem L, Vervelde L, Summers KM, et al. Characterization of Subpopulations of Chicken Mononuclear Phagocytes That Express TIM4 and CSF1R. *J Immunol.* 2019;202(4):1186-99.

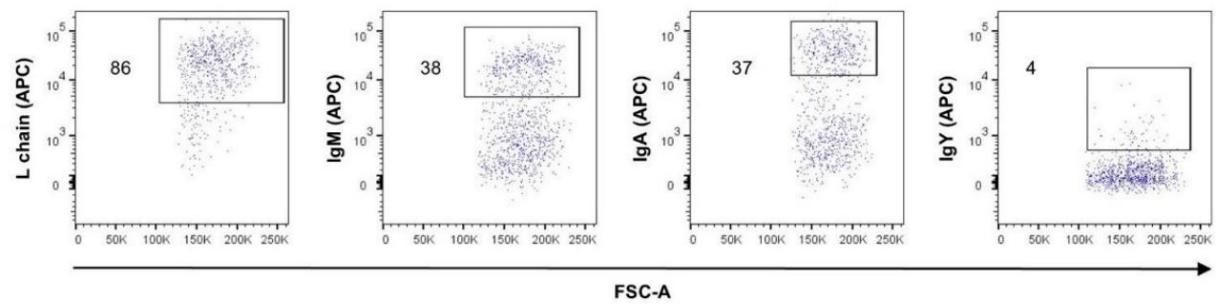
**Supplementary Figure 1**

Isotype controls for surface stainings on B-, T- and Plasma cells are shown for the respective antibody isotypes used within this study. Plots are gated for viable single cells

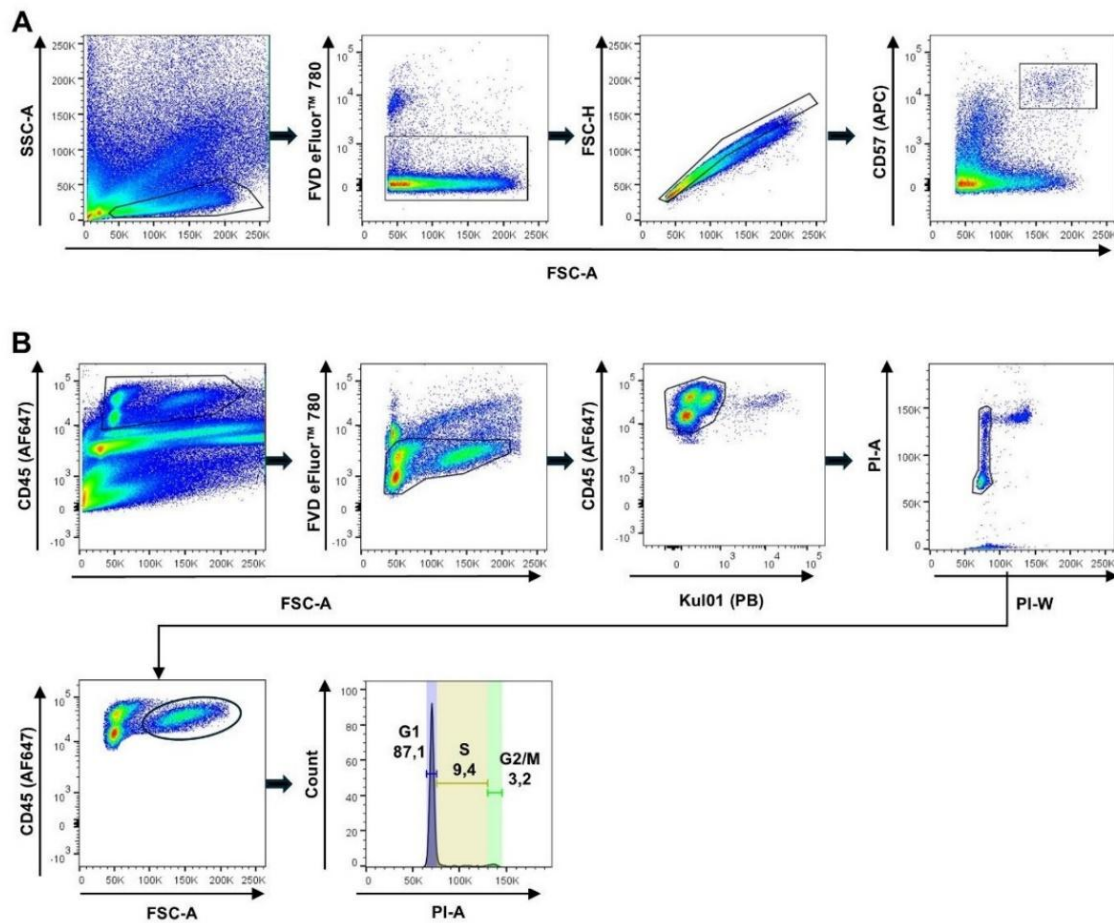


**Supplementary Figure 2**

Histograms showing the proportions of specific markers within the T cell population. The data shown is representative of three independent experiments.

**Supplementary Figure 3**

*Shares of surface light chain<sup>+</sup> and surface immunoglobulin<sup>+</sup> fractions within plasma cells defined as large CD45<sup>+</sup> are depicted.*



#### Supplementary Figure 4

(A) Depicts the gating strategy employed for surface Ig staining, defining plasma cells as large CD57<sup>+</sup> cells (Figure 4C), beginning with an initial gate on leukocytes, followed by the exclusion of dead cells and the selection of single cells. Subsequently, cells were displayed as CD57<sup>+</sup> plotted against size. (B) Shows the gating strategy for cell cycle analysis, starting with a gate on CD45-expressing leukocytes, followed by the exclusion of dead cells and macrophages/monocytes. In the final step, doublets were excluded. The gate for subpopulations was set in a CD45 vs. FSC-A plot, and cell cycle phases were determined using the FlowJo 10.10.0 cell cycle tool.

## 6 Diskussion

Trotz ihrer zentralen Rolle als antikörpersezernierender Arm in der humoralen Immunantwort lagen bislang nur wenige Erkenntnisse zu Plasmazellen beim Huhn vor. Ein besseres Verständnis dieser Zellen ist aber essentiell zur gezielten Entwicklung neuer und Modulation vorhandener Impfstoffe um die Impfantikörperantwort steuern und verbessern und die Tiere so besser schützen zu können. In dieser Arbeit wurden daher Isolationsprotokolle für Hühner-Plasmazellen entwickelt und diese Zellen näher charakterisiert.

Nachdem eine markerbasierte Isolation der Zellen mangels spezifischer Antikörper bisher nicht möglich war und histologischen Untersuchungen lediglich Hinweise lieferten, dass Plasmazellen den Hühner-B-Zell-Marker chB6 herunterregulieren (240) wurden dafür zwei verschiedene Ansätze, ein *in-vitro* und ein *ex-vivo* Ansatz, gewählt.

### 6.1 Versuchsansätze

Zunächst wurde ein Kultursystem mit Milzleukozyten entwickelt, um B-Zellen mittels Stimulation mit CD40L und IL-10 in Kultur zu Plasmazellen auszudifferenzieren.

Als Grundlage dienten hierbei vorliegenden Daten, welche zeigen, dass eine Stimulation von Hühner B-Zellen mit CD40L alleine Proliferation, sowie Klassenwechsel von IgM zu IgY, den Verlust von chB6 und eine allgemein höhere Antikörpersekretion induziert (242), alles Zeichen eines Differenzierungsprozesses Richtung Plasmazelle.

Zusätzlich stand für das Huhn inzwischen rekombinantes IL-10 zur Verfügung, für welches aus der Säugerliteratur bekannt ist, dass eine Kombination mit CD40L eine Differenzierung von B-Zellen Richtung Plasmazelle induziert (151).

Bei Milzzellen des Huhnes zeigte die Kombination von CD40L und IL-10 einen starken synergistischen Effekt auf die Zellproliferation, wobei in den ersten Tagen der Kultur fast ausschließlich B-Zellen proliferierten. Zusätzlich konnte bereits nach zwei Tagen ein Verlust der Expression von chB6, sowie des BAFF-R festgestellt werden und nach vier Tagen ein deutlicher Immunglobulinklassenwechsel, vor allem zu IgY, aber auch hin zu

IgA. Das Kultursystem mit einer Kombination von CD40L und IL-10 ist folglich in der Lage, B-Zellen des Huhns wie beim Säuger zu Plasmazellen auszudifferenzieren.

Da es jedoch wichtig ist zu wissen, wo Plasmazellen im Huhn lokalisiert sind und auch unklar ist, inwieweit *in-vitro* generierte Zellen den im Gewebe entstehenden entsprechen, wurden Leukozytenpopulationen verschiedener Organe analysiert. Aufgrund fehlender, zellspezifischer Marker, wurde hierbei gezielt nach klassengewechselten Zellen gesucht, da diese bereits ein Differenzierungsprogramm durchlaufen haben mussten.

In Leukozyten aus Milz, Blut und dem Knochenmark konnten nur wenige klassengewechselte Zellen gefunden werden. Größere Mengen fanden sich aber in den untersuchten mukosalen Immunorganen, den Caecaltonsillen und der Harderschen Drüse. Dabei war auffällig, dass die zu IgA klassengewechselten Zellen der Caecaltonsille mit chB6 und dem BAFF-R noch B-Zell-Marker exprimierten, also einem früheren oder anderen Differenzierungsstadium entsprachen. Die klassengewechselten Zellen der Harderschen Drüse hingegen waren mehrheitlich wesentlich größer als die im Organ gefundenen nicht-klassengewechselten B-Zellen und exprimierten weder chB6 noch den BAFF-R.

Nachdem für die Hardersche Drüse bereits Beschreibungen zu einer möglichen Isolation mittels Dichtegradienten-Zentrifugation vorlagen (293) und die durchflusszytometrisch untersuchten klassengewechselten Zellen als große, B-Zell-Marker-negative, Immunglobulin-positive Zellen einem erwarteten Plasmazellphänotyp entsprachen, wurde für diese ein markerbasiertes (FSC-A<sup>high</sup>/sIg<sup>+</sup>/CD45<sup>+</sup>) Isolationsprotokoll etabliert.

Somit standen nun zwei Quellen zur Verfügung, die eine nähere Charakterisierung von Plasmazellen ermöglichten.

## 6.2 Phänotyp der *in-vitro* generierten Plasmazellen

Zunächst wurden die in Kultur proliferierenden Zellen hinsichtlich ihrer Expression der Aktivitätsmarkern CD25, als hoch-affine Kette des IL-2 Rezeptors und CD80, als B-Zell-assoziiertes Aktivierungsantigen (294), untersucht. Im Verlauf der Kultivierung konnte für beide Marker eine initial deutliche Steigerung der Expression mit anschließend starker Abnahme gezeigt werden. Während bei frisch isolierten Zellen der Anteil von CD25-exprimierenden Zellen etwa ein Drittel der B-Zellen ausmachte und CD80 kaum zu



detektieren war, zeigte sich nach zwei Tagen in Kultur eine deutliche Hochregulation beider Marker. Zu späteren Zeitpunkten verschwand die Expression von CD25 fast vollständig und die Expression von CD80 wurde deutlich herunterreguliert.

Da CD25 beim Säuger nur auf einem geringen Anteil der naiven B-Zellen vorkommt und seine Expression ansonsten hauptsächlich mit B-Gedächtniszellen assoziiert ist (295), legt die biphasische Expression von CD25 auf stimulierten Hühner-B-Zellen nahe, dass es trotz der durchgehenden Stimulation mit CD40L und IL-10, entweder zwei klar abgegrenzte Phasen der Aktivierung gibt oder dass durch die Stimulation beim Huhn keine B-Gedächtniszellentwicklung induziert wird, sofern diese, wie die Gedächtniszellen beim Menschen (296), auch positiv für CD25 wären.

CD80 hingegen steht beim Säuger mit B-Zellen in der GC-Reaktion in Verbindung und wird auf Gedächtniszellen zum Teil und auf Plasmazellen nicht mehr exprimiert (297, 298). Wird also angenommen, dass dieses System vom Säuger auf das Huhn übertragbar ist, spricht dies dafür, dass das Kultursystem eine fortlaufende, sich abschwächende, GC-Reaktion nachstellt.

Zusammenfassend zeigen die Daten für CD25 und CD80, dass es im Kultursystem zu einer initialen Aktivitätsphase kommt, die mit der GC-Reaktion oder der initialen extrafollikulären Immunantwort vergleichbar ist und der eine Phase der Ausdifferenzierung folgt.

Anhand der Färbung mit einem Antikörper gegen die leichte Kette der Immunglobuline konnte unabhängig vom Immunglobulin-Isotyp nachgewiesen werden, dass die B-Zellen in Kultur durchgehend Oberflächenimmunglobulin exprimieren. Während dabei zu Beginn hauptsächlich Zellen vom IgM-Isotyp nachgewiesen wurden, zeigten sich zu späteren Zeitpunkten deutliche Populationen von zu IgA und IgY klassengewechselten Zellen, was die Zweiphasigkeit des Systems hinsichtlich einer initialen Aktivierungs- und späteren Differenzierungsphase, zusätzlich unterstreicht.

Ein weiterer Beleg für die durch das Kultursystem induzierte Plasmazelldifferenzierung ist die signifikante Zunahme der Anzahl antikörpersezernierender Zellen jedes Isotyps, die mittels ELISpot-Assay nachgewiesen wurden, sowie der deutliche Anstieg von  $\mu$ S, der löslichen Form von IgM, die durch qRT-PCR detektiert wurde.

Zudem zeigten Analysen der exprimierten Transkriptionsfaktoren innerhalb der proliferierenden Zellen eine starke Abnahme des für B-Zellen typischen Transkriptionsfaktors Pax-5, bei einer gleichzeitigen massiven Steigerung der Expression von Blimp-1, dem für Plasmazellen charakteristischen Transkriptionsfaktor (129).

Die durchflusszytometrische Charakterisierung der Expression von chB6 und BAFF-R, einem der beiden Rezeptoren für BAFF im Huhn, die beide bereits ab dem präbursalen Stadium auf Hühner-B-Zellen exprimiert werden und als Marker für ihre Identifizierung herangezogen werden, zeigte im Verlauf der Stimulation eine deutliche bis hin zur nahezu vollständigen Reduktion. Folglich können diese Zellen als „markernegativ“ bezeichnet werden. Die Reduktion der Expression von chB6, die auch mittels qRT-PCR nachweisbar war, bestätigt damit die bereits in deutlich weniger sensitiven histologischen Untersuchungen für Plasmazellen beschriebene fehlende chB6 Färbung (240). Die Reduktion der Expression von BAFF-R entspricht einem im Vergleich zum Säuger konservierten Mechanismus der Differenzierung, da auch dort die Reduktion im Rahmen der Differenzierung beschrieben ist (299).

Ein weiteres im Rahmen der durchflusszytometrischen Analysen untersuchtes Molekül ist CD57. Dieses, auch als HNK-1 bezeichnete, Glycoprotein wurde für das Huhn bereits als Aktivierungsmarker von T-Zellen (300) beschrieben. Zudem gab es erste Berichte, dass es sich auch um einen Aktivierungsmarker für B-Zellen und möglichen Plasmazellmarker handeln könnte (262). Mittels eines kreuzreaktiven humanen Antikörpers konnte, ähnlich wie in humanen B-Zellen (301), auf unstimulierten B-Zellen kaum CD57 nachgewiesen werden. Im Verlauf der Stimulation kam es jedoch zu einer deutlichen Expressionssteigerung, wobei ein Teil der Zellen interessanterweise durchgehend negativ blieb. Dieses Expressionsmuster war unabhängig vom Immunglobulinklassenwechsel und grenzt CD57 deutlich von anderen Aktivierungsmarkern wie CD25 oder CD80 ab.

Die in einem Kandidatengen-Ansatz in unserem Labor generierten Antikörper gegen hühnerspezifisches CD138 und TACI, beides Marker, die bei Mensch und Maus zur Identifizierung terminal differenzierten B-Zellen eingesetzt werden (100, 198, 302), zeigten initial eine starke Induktion der Expression. Im Verlauf der Stimulation nahm die Expression jedoch deutlich ab. Gegen Ende der Stimulationsperiode wurde für TACI nur noch sehr schwach und CD138 nur noch von wenigen Zellen exprimiert. Vor allem CD138

scheint sich daher, im Gegensatz zum Säuger, eher als Marker von Aktivierungsprozessen, wie sie zum Beispiel im GC stattfinden, als für terminal differenzierte Zellen zu eignen.

Für den Chemokinrezeptor CXCR4, der auf Plasmazellen von Säugern exprimiert ist und in Interaktion mit seinem Liganden CXCL12 für das „homing“ in Plasmazellnischen des Knochenmarks verantwortlich ist (303), zeigte sich eine deutliche Zunahme der Expression zu Beginn der Stimulation, die nachfolgend aber rasch annähernd auf das Ausgangsniveau unstimulierter B-Zellen zurückging. Dies spricht ebenfalls für eine Keimzentrums-ähnliche Aktivität in unserem Kultursystem, da beim Säuger gezeigt werden konnte, dass proliferierende Keimzentrums-B-Zellen CXCR4 hochregulieren, um in der CXCL12-reichen dunklen Zone des Keimzentrums zurückgehalten zu werden (51). Im Zuge ihrer finalen Differenzierung verlieren Plasmablasten anschließend CXCR4, wodurch ihnen das Verlassen des Keimzentrums ermöglicht wird.

### **6.3 Plasmazellen der Harderschen Drüse**

Im Rahmen der Etablierung des Isolationsprotokolls von Plasmazellen aus der Harderschen Drüse wurden die Zellen zunächst mit dem Pan-Leukozyten-Marker CD45 angefärbt. So konnte eine Population CD45 exprimierender Zellen identifiziert werden, die deutlich größer als die enthaltenen B- und T-Lymphozyten war. Allerdings enthielt diese Population auch einen kleinen Anteil Makrophagen, die aber für weitere Analysen, markerbasiert ausgeschlossen werden konnten.

Zytospinpräparate von durchflusszytometrisch aufgereinigten Zellen konnten für diese Population den plasmazelltypischen Phänotyp (53) von großen Zellen, die einen dezentralen Nucleus mit Wagenradstruktur, sowie basophiles Zytoplasma besitzen, nachweisen. Analysen des Transkriptionsfaktorprofils zeigten, dass diese Zellen hohe Spiegel des plasmazellspezifischen Blimp-1 enthalten. Somit konnte für die Population großer, CD45 exprimierender Zellen nachgewiesen werden, dass es sich, sofern Makrophagen ausgeschlossen werden, um Plasmazellen handelt. Die Expression von CD45 auf der Zelloberfläche der Plasmazellen stellt einen weiteren Unterschied zum Säuger da, bei dem für LLPCs in den Nischen des Knochenmarks und Darms CD45-Populationen beschrieben werden (304-306).

Weitere durchflusszytometrische Analysen, mittels Färbung der leichten Kette, konnten zeigen, dass alle Zellen immunglobulinpositiv waren.

Die Zuordnung der Isotypen gestaltete sich zunächst schwierig, da die Summe der einzelnen Isotypen, hauptsächlich IgM und IgA bei nur wenig IgY, zunächst nicht den Anteil der Zellen ergab, der sich für die leichte Kette anfärben ließ. Außerdem hatten immunhistologische Färbungen neben IgM und IgA positiven Zellen auch eindeutig einen größeren Anteil IgY exprimierender Zellen ausgewiesen, der in der Durchflusszytometrie zunächst fehlte. Interessanterweise zeigte sich diese Diskrepanz aber nicht, wenn die Zellen nach der CD45-Färbung permeabilisiert und die Immunglobuline intrazellulär angefärbt wurden. Hierbei ließen sich deutlich mehr IgY positive Zellen anfärben und die Summe aus IgM, IgA und IgY entsprach dem Anteil an L-Ketten positiven Zellen.

Da weitere Untersuchungen der Plasmazellpopulation zeigten, dass diese auch mit dem in der Kultur zuvor erfolgreich getesteten CD57 Antikörper anfärbbar waren, wurden in einem weiteren Versuchsansatz die Plasmazellen statt mit CD45 als große CD57 exprimierende Zellen angesprochen. In dieser Färbung gelang es, auch in der Oberflächenfärbung deutlich mehr IgY positive Zellen nachzuweisen, sodass, die Summe der einzelnen Isotypen den L-Ketten positiven Zellen entsprach und die Ergebnisse in Übereinstimmung mit dem immunhistologischen Färbebild waren. Als wahrscheinlichste Ursache kann für diese Beobachtung eine sterische Hinderung zwischen der Antikörperbindung an CD45 und dem B-Zellrezeptor vom IgY-Isotyp angenommen werden, da das CD45-Molekül auf der Zelloberfläche in räumlicher Nähe zum B-Zellrezeptor liegt und in großer Anzahl in der Membran vorhanden ist.

Von den im Kandidatengen-Ansatz hergestellten Antikörpern konnten für CD138 keine positiven Zellen nachgewiesen werden, so dass festgehalten werden kann, dass sich der Antikörper, wie schon im *in-vitro* Ansatz gezeigt, nicht als Marker ausdifferenzierter Plasmazellen eignet. TACI hingegen wurde auf allen Zellen, wenn auch nur niedrigschwellig, nachgewiesen.

Untersuchungen der Expression von chB6 und BAFF-R zeigten, dass die Plasmazellen der Harderschen Drüse für beide Marker negativ sind. Somit konnte mit dem Verlust von chB6 der histologisch beschriebene Phänotyp von Hühner-Plasmazellen bestätigt werden

(240). Ebenso scheint der Mechanismus des BAFF-R Verlustes im Rahmen der Plasmazell-Differenzierung zwischen Säuger und Huhn konserviert.

Der Chemokinrezeptor CXCR5, dessen Expression im Rahmen der Plasmazelldifferenzierung beim Menschen deutlich herunterreguliert wird (307), offenbarte innerhalb der Plasmazellen der Harderschen Drüse interessanterweise zwei distinkte Populationen (CXCR5<sup>+</sup> und CXCR5<sup>-</sup>) und damit eine deutliche Diskrepanz zu den einheitlich negativen Zellen beim Säuger. Dies lässt eine klare Trennung der Empfänglichkeit über CXCL13-vermittelte Signale erkennen und könnte auf unterschiedliche Funktionen der beiden Populationen hindeuten.

#### **6.4 Vergleich von *in-vitro* generierten und *ex-vivo* isolierten Plasmazellen**

Sowohl in dem beschriebenen *in-vitro* als auch im *ex-vivo* Ansatz ist es gelungen, Plasmazellen zu generieren beziehungsweise zu isolieren, die im Folgenden verglichen werden:

Hinsichtlich der Expression des BCR konnte gezeigt werden, dass in beiden Ansätzen Plasmazellen jeden Isotyps generiert beziehungsweise isoliert wurden, auch wenn innerhalb der klassengewechselten Zellen im *in-vitro* Ansatz IgY und *ex-vivo* IgA dominierten. Da *ex-vivo* mit einem mukosalen Immunorgan gearbeitet wurde, ist es jedoch nicht verwunderlich, dass IgA, als dominantes Immunglobulin mukosaler Oberflächen, deutlich stärker vorhanden war. Vergleichend zum Säuger zeigen sich hinsichtlich der BCR-Expression aber überraschende Unterschiede, da beim Säuger zwar sowohl für IgM- als auch IgA-Plasmazellen beschrieben ist, dass sie weiterhin einen funktionellen BCR auf der Oberfläche exprimieren, bei IgG<sup>+</sup> Plasmazellen allerdings kaum noch Immunglobuline und damit B-Zellrezeptoren auf der Oberfläche exprimiert werden (308-310). Dies weist auf unterschiedliche Funktionen hinsichtlich ihrer Aktivierbarkeit hin, da es beim Huhn zu einer direkten Interaktion von IgY<sup>+</sup>-BCRs mit dem Antigen kommen kann.

chB6 als Marker von B-Zellen wurde, ebenso wie der BAFF-R, in beiden Ansätzen herunterreguliert. So konnte eindeutig gezeigt werden, dass die Plasmazellen des Huhns als markernegative Zellen anzusprechen sind.



Bei den Aktivierungsmarkern entsprach die starke Herunterregulation von CD25 am Ende der Stimulationsperiode den Plasmazellen der Harderschen Drüse, von denen nur ein sehr geringer Anteil CD25 exprimiert. Die schwache Expression von CD80 gegen Ende auf *in-vitro* generierten Plasmazellen, spiegelte sich ebenfalls in den Plasmazellen der HG, die sich als schwach CD80<sup>+</sup> zeigten. Unterschiede konnten hingegen hinsichtlich der Expression von CD57 beobachtet werden. Während aus der Harderschen Drüse isolierte Plasmazellen vollständig positiv waren, zeigte sich in Kultur ein Teil der Zellen als negativ. Dies weist darauf hin, dass es sich bei den in Kultur entstehenden Zellen nicht um eine homogene Population handelt, sondern sich neben Plasmazellen noch andere Zellen, möglicherweise Gedächtniszellen differenzieren könnten. Auch wenn die Plasmazellen der Harderschen Drüse sich hinsichtlich des Expressionsmusters der Aktivierungsmarker, ebenso wie anderer Marker, sehr homogen darstellen, konnte mittels Zellzyklusanalysen nachgewiesen werden, dass sich etwa 10% der Zellen in der S-Phase des Zellzyklus befanden, also aktiv DNA replizieren und sich damit auf eine Zellteilung vorbereiten. Dies bedeutet, dass es sich bei der Population nicht um eine reine Population ausdifferenzierter Plasmazellen handelt, sondern, dass sie auch etwa 10% noch teilungsfähiger Plasmablasten enthält.

Dafür, dass es sich bei einem Großteil der Zellen in der HG um vollständig ausdifferenzierte, post-GC Plasmazellen handeln muss, spricht die im Vergleich zu B-Zellen deutliche niedrigere Expression von MHCII, welcher im Rahmen der Ausdifferenzierung herunterreguliert wird (311, 312). Hierdurch wird die Fähigkeit der Zellen Antigene präsentieren zu können, reduziert. Zudem zeigt die gesamte Zellpopulation eine deutlich erhöhte Expression der Chemokinrezeptors CXCR4. Das mittels qRT-PCR in der HG auch hohe Spiegel des entsprechenden Liganden CXCL12 festgestellt wurden (Daten nicht im Manuskript enthalten) deutet darauf hin, dass die Mikroumgebung der Harderschen Drüse für Plasmazellen eine Überlebensnische analog zu den Nischen im Knochenmark und Darm darstellen könnte und die CXCR4-CXCL12-Achse die Zellen nicht nur in die Nische leitet, sondern auch Überlebenssignale vermittelt (72, 303). Im Gegensatz hierzu spricht die Reduktion von CXCR4 in Kultur dafür, dass es sich um eine Nachstellung der GC-Reaktion handelt, in der nicht vollständig ausdifferenzierte Plasmablasten die Expression von CXCR4 reduzieren, um zwischen heller und dunkler Zone zu wandern beziehungsweise um das GC zu verlassen.

Beim Säuger wird das Überleben von Plasmazellen u.a. durch die Bindung von APRIL an BCMA (dem dritten Rezeptor für BAFF neben BAFF-R und TACI) vermittelt (313). Das BAFF/BAFF-R System beim Huhn ist jedoch mit BAFF als alleinigem Ligand und BAFF-R und TACI als Rezeptoren (bei BCMA handelt es sich um ein Pseudogen) einfacher gestaltet. Damit stellt sich hinsichtlich der sehr schwachen Expression von TACI gegen Ende der Kultur und der schwachen Expression auf Plasmazellen der Harderschen Drüse, die Frage, wie Plasmazellen des Huhns ausreichende Überlebenssignale erhalten (253, 254). Einerseits wäre es möglich, dass auch ohne April über BAFF und TACI alleine ausreichend Überlebenssignale vermittelt werden, andererseits wäre es auch möglich, dass noch weitere Faktoren, wie zum Beispiel CXCL12 das in der Harderschen Drüse deutlich exprimiert wird, oder IL-6, welches in den Knochenmarksnischen des Säugers eine Rolle spielt (72), von Bedeutung sind.

## 6.5 Markerprofil von (ausdifferenzierten) Plasmazellen

Im Rahmen dieser Arbeit ist es erstmalig gelungen, Hühner-Plasmazellen näher zu charakterisieren. Damit kann die Liste der Differenzierungsmarker für B-Zellen des Huhns folgendermaßen ergänzt werden:

**Tabelle 3** Ergänzte Übersicht der ansprechbar Oberflächenantigene auf Hühner-B-Zellen in verschiedenen Entwicklungsphasen nach Härtle et al. (256)

| Entwicklungsphase     | Antigene                                                                                                                                                                             | Literatur                                 |
|-----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------|
| prä-bursale B-Zellen  | chB6 ( <i>ab ED10</i> ), BAFF-R ( <i>ab ED14</i> )<br>CXCR4/5 ( <i>in der Milz</i> ), Sialyl-Lewis <sup>x</sup><br>(CD15s)                                                           | (208, 256-260)                            |
| bursale B-Zellen      | chB6, BAFF-R, MHCII, CD40, CXCR4,<br>CXCR5, Wechsel von CD15s zu CD15                                                                                                                | (208, 217, 243,<br>255, 256, 258-<br>260) |
| emigrierende B-Zellen | Ig Light chain, MHCII, chL12, CXCR4 <sup>low</sup>                                                                                                                                   | (208, 218, 256)                           |
| periphere B-Zellen    | CD5, chB6, BAFF-R, Ig Light chain,<br>MHCII, CD40                                                                                                                                    | (242, 245, 255,<br>256, 258, 261)         |
| GC B-Zellen           | <i>keine spezifischen Marker vorhanden</i><br><i>möglicherweise CD57</i>                                                                                                             | (256, 262)                                |
| B-Gedächtniszellen    | <i>keine spezifischen Marker vorhanden</i>                                                                                                                                           | (256)                                     |
| Plasmazellen          | intrazelluläres Immunglobulin<br><b>Oberflächenimmunglobulin (alle Isotypen)</b><br><b>chB6<sup>+</sup>/BAFF-R<sup>+</sup></b><br><b>CD57<sup>+</sup></b><br><b>CD45<sup>+</sup></b> | (240, 242, 256,<br>262)                   |

## 6.6 Ergänzende Ergebnisse und weiterführende Überlegungen

Interessanterweise konnte gezeigt werden, dass die Involution der Bursa zu Beginn der Legereife keinen negativen Einfluss auf die Frequenz von B-Zellen in Blut und Milz hatte. Im Gegenteil nahm der Anteil IgM<sup>+</sup>/Marker<sup>+</sup>-Zellen sogar zu. Dies verwundert zunächst, da mit der Bursa, das primäre lymphatische Organ, das für die Entwicklung und Reifung der B-Lymphozyten zuständig ist, verloren geht und daher davon ausgegangen werden könnte, dass dies einen negativen Einfluss auf die Frequenz von B-Zellen hat. Jedoch konnte bereits in anderen Versuchen eine Population von B-Zellen im Blut nachgewiesen werden, die aus sich schnell teilenden Zellen stammt, deren Quelle nicht die Bursa sein konnte, da die Tiere in diesen Versuchen bursektomiert waren (218). Die hierfür verantwortlichen Zellen sind vermutlich auch jene Zellen, die für die B-Zellproduktion nach der Bursainvolution verantwortlich sind und werden in der Milz vermutet, von der bekannt ist, dass sie eine Population sogenannter „post-bursaler Stammzellen“ beherbergt, die zur Rekonstitution des peripheren B-Zell-Pools beitragen können (4, 223). Ein kontinuierlicher Anstieg klassengewechselter Zellen konnte in diesen Untersuchungen nicht nachgewiesen werden und die punktuellen Peaks markernegativer Zellen stehen vermutlich eher mit den genutzten Impfschemata in Zusammenhang.

Neben dem Einfluss des Alters wurde auch der Einfluss einer Impfung gegen das IBD-Virus (Infectious Bursa Disease Virus) untersucht. Die Impfung wurde mit einem attenuierten Lebendimpfstoff durchgeführt, der zu einer Depletion der B-Zellen in der Bursa, gefolgt von einer anschließenden Regeneration des Organs (314) führt. Gleichzeitig induzierte der Impfstoff eine starke Antikörperantwort gegen IBDV (315), was unserer Annahme nach mit einer Zunahme klassengewechselter B-Zellen in Verbindung stehen würde. Vier Wochen nach der Impfung konnte tatsächlich ein signifikanter Anstieg des Anteils klassengewechselter Zellen festgestellt werden, ebenso wie eine signifikante Zunahme der markernegativen, nicht klassengewechselten Zellen. Mögliche Erklärungen für diese Beobachtung sind, dass entweder die vorhandenen differenzierten Zellen eine höhere Resistenz gegen das IBD-Virus haben und daher nicht durch die Virusinfektion eliminiert wurden. Ferner wäre es möglich, dass durch die Immunantwort gegen das IBD-Virus IBDV-spezifische, differenzierte B-Zellen generiert. Um die Ursachen des beobachteten Effektes zu klären, sind weitere detaillierte Untersuchungen dieser Zellen nötig. Diese sollten auf jeden Fall eine absolute Quantifizierung der Zellen enthalten, um

abzuklären, ob es sich um eine absolute oder relative Zunahme der Zellen handelt. Darüber hinaus wäre es von Interesse, markerpositive und markernegative klassengewechselte Zellen hinsichtlich eines möglichen Memory-Phänotyps eingehender zu charakterisieren, da dieser Zelltyp beim Huhn bislang nicht beschrieben wurde, er aber nach Impfung entstehen bzw. sein Anteil zunehmen sollte.

## **6.7 Ausblick**

Die Erkenntnisse, die innerhalb dieser Arbeit gewonnen werden konnten, führen zu einer Vielzahl von Anwendungsmöglichkeiten, die im Folgenden weiter diskutiert werden.

### **6.7.1 Mögliche Optimierungen des Kultursystems**

Innerhalb des Kultursystems mit CD40L und IL-10 konnte sowohl auf der Ebene von Oberflächenmarkern als auch auf Transkriptionsfaktorebene neben einer initialen Aktivierungsphase (Expressionssteigerung von CD25, CD80, CD57, CD138, TACI) eine zweite Phase der weiteren Differenzierung (Abnahme der Expression von chB6 und BAFF-R, sowie hohe Blimp-1-Level und Immunglobulin-Klassenwechsel) nachgewiesen werden.

In Zellkulturversuchen mit naiven humanen B-Zellen sowie GC-B-Zellen wurde gezeigt, dass für eine terminale Differenzierung zur Plasmazelle ein Beenden des CD40L-Signals nötig ist (151, 316) und ein Beibehalten eher zur Differenzierung in Richtung eines Gedächtniszell-Phänotyps führt (151). Daher sollte in zukünftigen Versuchen CD40L nach einer ersten Phase der Aktivierung aus der Kultur entfernt werden, um zu prüfen ob auch beim Huhn dadurch die terminale Differenzierung der Zellen in Richtung Plasmazelle gefördert werden kann.

Zusätzlich könnten neben CD40L und IL-10 noch weitere Zytokine, wie das für die GC-Reaktion essenzielle IL-21, in die Kultur eingebracht werden. IL-21 induziert bei CD40-stimulierten Säuger B-Zellen sowohl eine starke Proliferation als auch die Differenzierung in Richtung eines Plasmazell-Phänotyps und steigert nachweislich die Antikörperproduktion (317, 318). Obwohl die Rolle von IL-10 und IL-21 in der GC-Reaktion redundant erscheint, wirken sie in unterschiedlichen Differenzierungsstadien unterschiedlich effizient: IL-21 verstärkt die Initiationsphase, während IL-10 die Differenzierung von Plasmablasten zu Plasmazellen fördert (177). Diese Erkenntnisse

legen nahe, dass es möglicherweise sinnvoll wäre, das Kultursystem über die zeitlich versetzte Zugabe von IL-21 und IL-10 zu optimieren.

Die in den Kulturen anfangs beobachtete starke Hochregulation von CD25, der  $\alpha$ -Kette des IL-2-Rezeptors, könnte darauf hinweisen, dass sich differenzierende B-Zellen sensibel auf IL-2 reagieren. In diesem Zusammenhang wäre es sinnvoll zu prüfen, ob die Zugabe von IL-2 die Differenzierung weiter beeinflusst. Interessanterweise zeigen neuere Studien von Sun et al., dass CD25 auf B-Zellen eher eine Feedback-Funktion für die BCR-Signalübermittlung übernimmt, anstatt als funktionelle IL-2-Rezeptorkomponente zu wirken (319). Diese Erkenntnis legt nahe, dass CD25 eine komplexere Rolle in der B-Zell-Biologie spielt, als bisher angenommen, da die CD25-Expression auf B-Zellen bisher hauptsächlich mit einem B-Gedächtniszell-Phänotyp und dessen besserer Antigenpräsentations- und Proliferationskapazität in Verbindung gebracht wurde (296, 320-323).

Um den Einfluss von Zytokinen gezielt untersuchen zu können wäre es sinnvoll, weitere Versuche mit, durch Sortierverfahren wie FACS (Fluorescence Activated Cell Sorting) oder MACS (Magnetic Activated Cell Sorting), aufgereinigten B-Zellen durchzuführen, da wir in unserem Kultursystem zeigen konnten, dass es zu späteren Zeitpunkten zu einer starken Proliferation von, vor allem CD4-positiven T-Zellen kommt. Es kann also nicht ausgeschlossen werden, dass im bisherigen auf einer gemischten Ausgangspopulation basierenden Kultursystem bereits IL-21 vorhanden war, da die stark proliferierende T-Zellen zu späteren Zeitpunkten potenziell IL-21 sezernieren.

Ebenso kann die Anwesenheit von IL-2 nicht ausgeschlossen werden, da für CD40-aktivierte B-Zellen beim Menschen und Kaninchen gezeigt wurde, dass sie autokrin IL-2 produzieren (324). Sollte dies auch bei Hühner-B-Zellen der Fall sein, könnte dies eine Erklärung für die beobachteten Differenzierungsprozesse und die zelluläre Zusammensetzung des Kultursystems liefern.

### **6.7.2 Kandidatengen-Ansätze**

Die in Kultur und *ex-vivo* analysierte Expression, der in Kandidatengen-Ansätzen gewonnenen Antikörper gegen TACI und CD138, kann hinsichtlich der Identifikation von Plasmazellen als teilerfolgreich bezeichnet werden, da zumindest für TACI eine niedrige Expression auf Plasmazellen nachgewiesen werden konnte. Im Gegensatz dazu war

CD138 auf den Plasmazellen nicht nachweisbar und scheint beim Huhn eher ein Marker von Aktivierungsprozessen innerhalb der GC-Reaktion zu sein. Hier konnte eindrucksvoll gezeigt werden, dass nicht alle Erkenntnisse hinsichtlich der Expression von Oberflächenantigenen aus dem Säuger auf das Huhn übertragbar sind, sondern fundamentale Unterschiede bestehen und jeder Marker individuell überprüft werden muss.

Nachdem allerdings durch die erfolgreiche Isolation aus der HG nun ein direkter Zugriff auf Plasmazellen des Huhns möglich ist, kann jetzt gezielt nach weiteren möglichen Kandidatengen innerhalb der Plasmazell-Population des Huhns gesucht werden, die dann für die Generierung neuer Zell-spezifischer Marker genutzt werden können. Hierfür stehen Sequenzierungsmethoden für ganze Zellpopulationen oder für Einzelzellen, zur Verfügung, die die Untersuchung der gesamten Transkriptomlandschaft einer Zellpopulation oder der Genexpression auf Einzelzellebene (scRNASeq) ermöglicht. Letztere bietet eine hohe Auflösung und ist besonders nützlich zur Identifizierung seltener Zelltypen oder Subpopulationen, die in der Bulk-Sequenzierung möglicherweise übersehen werden.

Da wir für die Plasmazellen der Harderschen Drüse zeigen konnten, dass es sich um eine heterogene Population handelt, sowohl hinsichtlich des Zellzyklus, als auch hinsichtlich der Expression verschiedener Ig-Isotypen, wäre scRNASeq möglicherweise die interessantere Methode, da hier auch Unterschiede zwischen den verschiedenen Plasmazellen (IgM vs. IgA vs. IgY) festgestellt werden könnten.

Ebenso bieten die Sequenzierungsmethoden eine gute Möglichkeit, Zellpopulationen näher zu untersuchen, die im Rahmen dieser Arbeit nicht näher charakterisiert werden konnten. Dazu gehören beispielsweise die markernegativen, Ig-positiven Zellen, die in der Harderschen Drüse gefunden werden konnten, bei denen über Genexpressionsanalysen das Vorhandensein von für Memory-Zellen spezifischen Genen untersucht werden könnte.

### **6.7.3 Impfstoffentwicklung**

Der Zugriff auf die Plasmazellen aus der Harderschen Drüse bietet nicht nur die Möglichkeit einer genaueren Untersuchung der Zellen, sondern bietet auch eine Möglichkeit die Wirkung von Impfstoffen beziehungsweise Mechanismen der auf



Impfungen folgenden Antikörperantwort genauer zu untersuchen. Die HG als lokales Immunorgan im Auge bietet sich hier besonders an, da Impfungen gegen das Newcastle-Disease-Virus (NDV), eine Pflichtimpfung für Hühner in Deutschland, oder Kombiimpfstoffe gegen NDV und IBV (Infektiöses Bronchitis Virus) über das Auge, in Form von Tropfen, verabreicht werden können.

Der direkte Zugriff auf die Antikörper-produzierenden Zellen der HG bietet eine wertvolle Ergänzung zu den bisherigen Untersuchungen der lokalen Immunantwort, die sich bisher hauptsächlich auf Untersuchungen von Antikörperspiegeln in der Tränenflüssigkeit beschränkten (325, 326).

Als mukosales Immunorgan bietet die Hardersche Drüse zusätzlich die Möglichkeit im Kontext der Impfstoffentwicklung verschiedene Adjunvantien zu testen, die vor allem die IgA-Antwort unterstützen sollen.

Zusammenfassend lässt sich festhalten, dass zwar noch viele Fragen, hinsichtlich der terminalen B-Zelldifferenzierung, sowie genauer Mechanismen der Antikörperproduktion und funktionaler Mechanismen offen sind, jedoch hat diese Arbeit die Grundlage geschaffen, einige dieser Fragen in Zukunft gezielt adressieren zu können.

## 7 Zusammenfassung

### **Isolierung und Charakterisierung von Plasmazellen des Haushuhns (*Gallus gallus*)**

Plasmazellen bilden das finale Differenzierungsstadium der B-Zell-Entwicklung und sind für die Antikörperproduktion verantwortlich. Obwohl beim Geflügel viele Impfstoffe ihre Schutzwirkung über die induzierte Antikörperantwort vermitteln, war über Plasmazellen des Huhnes bislang nur sehr wenig bekannt. Dies lag unter anderem darin begründet, dass außer ihres charakteristischen histologischen Phänotyps, keine Marker für ihre Identifikation zur Verfügung standen. Ziel dieser Arbeit war es daher, diese Zellen mittels verschiedener Ansätze näher zu charakterisieren.

Hierfür wurde zum einen ein *in-vitro* Kultursystem mit Milzleukozyten etabliert, bei dem die Stimulation mit den Zytokinen CD40L und IL10 zur Aktivierung der B-Zellen, ihrer Proliferation und letztlich zu ihrer Ausdifferenzierung in Richtung Plasmazellen führte. Dabei gelang es auch einen Immunglobulinklassenwechsel von IgM sowohl zu IgA als auch IgY zu induzieren. Als Nachweis der Plasmazelldifferenzierung dienten dabei u.a. die starke Zunahme der Zellgröße (FSC<sup>high</sup>) und Antikörpersekretion sowie die Expression des für Plasmazellen charakteristischen Transkriptionsfaktors Blimp-1 bei gleichzeitigem Verlust des für B-Zellen kanonischen Transkriptionsfaktors Pax-5. Die eingehende durchflusszytometrische Untersuchung der entstehenden Zellen zeigte, dass sie die für Hühner-B-Zellen typischen Marker chB6 und den Rezeptor für den B-Zellüberlebensfaktor BAFF, BAFF-R beinahe vollständig verloren hatten. Positiv waren sie dagegen für den Chemokinrezeptor CXCR4 und ein Großteil der Zellen exprimierte den Aktivierungsmarker CD57. Unabhängig davon, ob die Zellen weiter IgM bildeten oder einen Klassenwechsel zu IgY oder IgA durchlaufen hatten, exprimierten sie weiterhin membrangebundene Immunglobuline (also den B-Zellrezeptor) auf der Oberfläche. Dies ist besonders interessant, da IgG produzierende Plasmazellen des Säugers auf ihrer Oberfläche keinen B-Zellrezeptor mehr besitzen.

In einem weiteren Ansatz wurde ein Isolationsprotokoll für Plasmazellen direkt aus Gewebe etabliert. Hierzu diente die Hardersche Drüse (HG), eine Tränendrüse in der Augenhöhle, die histologisch eine große Anzahl von Zellen mit Plasmazell-Phänotyp aufwies.

Aus Präparationen von HG-Leukozyten gelang es, eine Population großer, CD45-positiver Zellen zu isolieren, die mikroskopisch Charakteristika von Plasmazellen aufwiesen, Blimp-1 exprimierten und Immunglobulin (zum großen Teil IgA) auf der Oberfläche trugen. Auch diese Zellen hatten die B-Zell typischen Marker (chB6, BAFF-R und Pax-5) verloren und waren positiv für CXCR4 und CD57, womit sie weitgehend den in Kultur differenzierten Zellen entsprachen.

Da humane Plasmazellen u.a. durch ihre Expression von CD138 und TACI (einem weiteren Rezeptor für BAFF charakterisiert sind), wurden zusätzlich Antikörper gegen hühnerspezifisches CD138 und TACI generiert. Obwohl beide Marker in den Kulturen kurzfristig induziert werden konnten, war TACI nur auf Zellen der HG in sehr geringen Mengen nachweisbar und CD138 weder auf den in Kultur induzierten noch auf den Plasmazellen der HG zu finden. Dies zeigt, dass eine Übertragung von bei Säugern gewonnenen Erkenntnissen auf das Immunsystem des Huhns nur sehr eingeschränkt möglich ist.

Damit ist es im Rahmen dieser Arbeit zum ersten Mal gelungen, auf verschiedenen Wegen Hühner-Plasmazellen zu gewinnen und diese als große CD45<sup>+</sup>/chB6<sup>-</sup>/BAFF-R<sup>-</sup>/Ig<sup>+</sup>/CD57<sup>+</sup> Zellen anzusprechen. Zudem konnten deutliche Unterschiede (IgY<sup>+</sup>-BCR, CD138<sup>-</sup>, CXCR5<sup>+/+</sup>) aber auch konservierte Mechanismen (BAFF-R<sup>-</sup>, Pax-5<sup>-</sup>, Blimp-1<sup>high</sup>) im Vergleich zum Säuger aufgezeigt werden. Diese Zellen können nun für weiterführende Untersuchungen wie z.B mittels Next generation sequencing sowie funktionelle Studien genutzt werden und werden damit zu einem besseren Verständnis der humoralen Immunantwort beim Huhn beitragen.

## 8 Summary

### **Isolation and characterization of plasma cells of the domestic chicken (*Gallus gallus*)**

Plasma cells represent the final differentiation stage of B cell development and are responsible for antibody production. Although many poultry vaccines mediate their protective effect via the induced antibody response, very little was known about chicken plasma cells. One of the reasons for this was that, apart from their characteristic histological phenotype, no markers were available for their identification. The aim of this work was therefore to characterize these cells in more detail using various approaches.

First, we established, an *in-vitro* culture system with spleen leukocytes, where stimulation with the cytokines CD40L and IL10 led to the activation of B cells, their proliferation, and eventually their differentiation into plasma cells. This protocol also succeeded in inducing an immunoglobulin class switch from IgM to both IgA and IgY. Evidence of plasma cell differentiation included a significant increase in cell size (FSC<sup>high</sup>) and antibody secretion as well as the expression of the plasma cell-specific transcription factor Blimp-1, along with the simultaneous loss of the canonical B cell transcription factor Pax-5. Detailed flow cytometric analysis of the emerging cells showed that they had almost completely lost the typical of chicken B cell markers chB6 and the receptor for the B cell survival factor BAFF, BAFF-R. However, they stained positive for the chemokine receptor CXCR4 and the majority of the cells expressed the activation marker CD57. Regardless of whether the cells continued to produce IgM or underwent a class switch to IgY or IgA, they still expressed membrane-bound immunoglobulins (i.e., the B cell receptor) on their surface. This is particularly interesting because IgG-producing plasma cells in mammals no longer carry a B cell receptor on their surface.

In another approach, an isolation protocol for plasma cells directly from tissue was established. The Harderian gland (HG), a lacrimal gland in the eye socket, which histologically showed a large number of cells with a plasma cell phenotype, was used for this purpose. Preparations of HG leukocytes succeeded in isolating a population of large CD45-positive cells that microscopically exhibited characteristics of plasma cells, expressed Blimp-1, and were positive for immunoglobulin (mostly IgA) on their surface.

These cells had also lost the typical B cell markers (chB6, BAFF-R, and Pax-5) and were positive for CXCR4 and CD57, thus largely corresponding to the cells differentiated in culture.

Since human plasma cells are characterized by their expression of CD138 and TACI (another receptor for BAFF), antibodies against chicken-specific CD138 and TACI were additionally generated. Although both markers could be transiently induced in cultures, TACI was detectable only in very small amounts on HG cells, and CD138 was not found on either the cultured or HG plasma cells. This shows that transferring findings from mammals to the chicken immune system is only possible to a limited extent.

In summary, within this work, for the first time, chicken plasma cells were successfully obtained in various ways and identified as large CD45<sup>+</sup>/chB6<sup>-</sup>/BAFF-R<sup>-</sup>/Ig<sup>+</sup>/CD57<sup>+</sup> cells. Furthermore, clear differences (IgY<sup>+</sup>-BCR, CD138<sup>-</sup>, CXCR5<sup>+/-</sup>) but also conserved mechanisms (BAFF-R<sup>-</sup>, Pax-5<sup>-</sup>, Blimp-1<sup>high</sup>) could be demonstrated in comparison to mammals. These cells can now be used for further investigations, such as next-generation sequencing and functional studies, contributing to a better understanding of the humoral immune response in chickens.

## 9 Literaturverzeichnis

1. Landwirtschaft BfEu. Geflügelhaltung: BMEL; 2024 [Available from: <https://www.bmel-statistik.de/landwirtschaft/tierhaltung/gefluegelhaltung>].
2. Bundesamt D-s. Pressemitteilung Nr. 104 vom 15. März 2024: statistisches Bundesamt; 2024 [Available from: [https://www.destatis.de/DE/Presse/Pressemitteilungen/2024/03/PD24\\_104\\_413.html](https://www.destatis.de/DE/Presse/Pressemitteilungen/2024/03/PD24_104_413.html)].
3. Davison F. The Importance of the Avian Immune System and Its Unique Features. In: Kaspers B, Schat KA, Göbel TW, Vervelde L, editors. Avian Immunology. 3 ed. United Kingdom: Academic Press, Elsevier Ltd.; 2022. p. 1-11.
4. Ratcliffe MJ, Härtle S. B cells, the bursa of Fabricius, and the generation of antibody repertoires. In: Kaspers B, Schat KA, Göbel TW, Vervelde L, editors. Avian Immunology. 3 ed. United Kingdom: Academic Press, Elsevier Ltd.; 2022. p. 71-99.
5. Ratcliffe MJH. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. Developmental and Comparative Immunology. 2006;30(1-2):101-18.
6. Glick B, Chang TS, Jaap RG. The Bursa of Fabricius and Antibody Production. Poultry Sci. 1956;35(1):224-5.
7. Health EPoA, Animal Welfare EURLfAI, Nielsen SS, Alvarez J, Bicout DJ, Calistri P, et al. Vaccination of poultry against highly pathogenic avian influenza - part 1. Available vaccines and vaccination strategies. EFSA J. 2023;21(10):e08271.
8. Melchers F. Checkpoints that control B cell development. J Clin Invest. 2015;125(6):2203-10.
9. Murphy K, Weaver C, Berg L. The Development of B and T Lymphocytes. Janeways Immunobiology. 10 ed. Canada: W.W. Norton & Company; 2022. p. 301-46.
10. Melchers F, Karasuyama H, Haasner D, Bauer S, Kudo A, Sakaguchi N, et al. The surrogate light chain in B-cell development. Immunol Today. 1993;14(2):60-8.
11. Harly C, Cam M, Kaye J, Bhandoola A. Development and differentiation of early innate lymphoid progenitors. J Exp Med. 2018;215(1):249-62.
12. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med. 1991;173(5):1213-25.
13. Melchers F, ten Boekel E, Yamagami T, Andersson J, Rolink A. The roles of preB and B cell receptors in the stepwise allelic exclusion of mouse IgH and L chain gene loci. Seminars in Immunology. 1999;11(5):307-17.
14. Casellas R, Shih TA, Kleinewietfeld M, Rakonjac J, Nemazee D, Rajewsky K, et al. Contribution of receptor editing to the antibody repertoire. Science. 2001;291(5508):1541-4.
15. Prak EL, Weigert M. Light chain replacement: a new model for antibody gene rearrangement. J Exp Med. 1995;182(2):541-8.
16. Nunez C, Nishimoto N, Gartland GL, Billips LG, Burrows PD, Kubagawa H, et al. B cells are generated throughout life in humans. J Immunol. 1996;156(2):866-72.
17. Akashi K, Kondo M, Cheshier S, Shizuru J, Gandy K, Domen J, et al. Lymphoid development from stem cells and the common lymphocyte progenitors. Cold Spring Harb Symp Quant Biol. 1999;64:1-12.



18. Bain G, Maandag EC, Izon DJ, Amsen D, Kruisbeek AM, Weintraub BC, et al. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell*. 1994;79(5):885-92.
19. Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature*. 1995;376(6537):263-7.
20. Brack C, Hirama M, Lenhard-Schuller R, Tonegawa S. A complete immunoglobulin gene is created by somatic recombination. *Cell*. 1978;15(1):1-14.
21. Ehlich A, Schaal S, Gu H, Kitamura D, Muller W, Rajewsky K. Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. *Cell*. 1993;72(5):695-704.
22. Nemazee D. Receptor editing in lymphocyte development and central tolerance. *Nat Rev Immunol*. 2006;6(10):728-40.
23. Chung JB, Silverman M, Monroe JG. Transitional B cells: step by step towards immune competence. *Trends Immunol*. 2003;24(6):343-9.
24. Cherukuri A, Cheng PC, Pierce SK. The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. *J Immunol*. 2001;167(1):163-72.
25. Wen L, Brill-Dashoff J, Shinton SA, Asano M, Hardy RR, Hayakawa K. Evidence of marginal-zone B cell-positive selection in spleen. *Immunity*. 2005;23(3):297-308.
26. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell*. 1997;90(6):1073-83.
27. Loder F, Mutschler B, Ray RJ, Paige CJ, Sideras P, Torres R, et al. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J Exp Med*. 1999;190(1):75-89.
28. Levine MH, Haberman AM, Sant'Angelo DB, Hannum LG, Cancro MP, Janeway CA, Jr., et al. A B-cell receptor-specific selection step governs immature to mature B cell differentiation. *Proc Natl Acad Sci U S A*. 2000;97(6):2743-8.
29. Tew JG, Wu J, Qin D, Helm S, Burton GF, Szakal AK. Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. *Immunol Rev*. 1997;156:39-52.
30. Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, et al. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science*. 2001;293(5537):2108-11.
31. Madry C, Laabi Y, Callebaut I, Roussel J, Hatzoglou A, Le Coniat M, et al. The characterization of murine BCMA gene defines it as a new member of the tumor necrosis factor receptor superfamily. *Int Immunol*. 1998;10(11):1693-702.
32. von Bulow GU, Bram RJ. NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. *Science*. 1997;278(5335):138-41.
33. Patke A, Mecklenbrauker I, Erdjument-Bromage H, Tempst P, Tarakhovsky A. BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. *J Exp Med*. 2006;203(11):2551-62.
34. Woodland RT, Fox CJ, Schmidt MR, Hammerman PS, Opferman JT, Korsmeyer SJ, et al. Multiple signaling pathways promote B lymphocyte stimulator dependent B-cell growth and survival. *Blood*. 2008;111(2):750-60.
35. Yu G, Boone T, Delaney J, Hawkins N, Kelley M, Ramakrishnan M, et al. APRIL and TALL-1 and receptors BCMA and TACI: system for regulating humoral immunity. *Nat Immunol*. 2000;1(3):252-6.

36. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity*. 2012;36(1):13-21.
37. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol*. 2006;7(3):293-301.
38. Baumgarth N. B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-Induced IgM Production. *Front Immunol*. 2016;7:324.
39. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science*. 2001;293(5537):2111-4.
40. Tangye SG. To B1 or not to B1: that really is still the question! *Blood*. 2013;121(26):5109-10.
41. Appelgren D, Eriksson P, Ernerudh J, Segelmark M. Marginal-Zone B-Cells Are Main Producers of IgM in Humans, and Are Reduced in Patients With Autoimmune Vasculitis. *Front Immunol*. 2018;9:2242.
42. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 1999;99(1):23-33.
43. Gatto D, Wood K, Brink R. EBI2 operates independently of but in cooperation with CXCR5 and CCR7 to direct B cell migration and organization in follicles and the germinal center. *J Immunol*. 2011;187(9):4621-8.
44. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev*. 2009;229(1):152-72.
45. Reif K, Ekland EH, Ohl L, Nakano H, Lipp M, Forster R, et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature*. 2002;416(6876):94-9.
46. Cozine CL, Wolniak KL, Waldschmidt TJ. The primary germinal center response in mice. *Curr Opin Immunol*. 2005;17(3):298-302.
47. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity*. 2007;27(2):190-202.
48. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*. 2000;102(5):553-63.
49. Yu K. AID function in somatic hypermutation and class switch recombination. *Acta Biochim Biophys Sin (Shanghai)*. 2022;54(6):759-66.
50. MacLennan IC. Germinal centers. *Annu Rev Immunol*. 1994;12:117-39.
51. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol*. 2004;5(9):943-52.
52. Odendahl M, Mei H, Hoyer BF, Jacobi AM, Hansen A, Muehlinghaus G, et al. Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood*. 2005;105(4):1614-21.
53. Ribourtout B, Zandecki M. Plasma cell morphology in multiple myeloma and related disorders. *Morphologie*. 2015;99(325):38-62.
54. Elsner RA, Shlomchik MJ. Germinal Center and Extrafollicular B Cell Responses in Vaccination, Immunity, and Autoimmunity. *Immunity*. 2020;53(6):1136-50.

55. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature*. 2008;453(7195):667-71.
56. Weisel F, Shlomchik M. Memory B Cells of Mice and Humans. *Annu Rev Immunol*. 2017;35:255-84.
57. Bortnick A, Allman D. What is and what should always have been: long-lived plasma cells induced by T cell-independent antigens. *J Immunol*. 2013;190(12):5913-8.
58. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. *Nat Rev Immunol*. 2015;15(3):160-71.
59. Auner HW, Beham-Schmid C, Dillon N, Sabbattini P. The life span of short-lived plasma cells is partly determined by a block on activation of apoptotic caspases acting in combination with endoplasmic reticulum stress. *Blood*. 2010;116(18):3445-55.
60. Zinkernagel R. On plasma cell longevity or brevity. *Expert Rev Vaccines*. 2014;13(7):821-3.
61. Minges Wols HA. Plasma Cells. eLS2006.
62. Tiburzy B, Szyska M, Iwata H, Chrobok N, Kulkarni U, Hirose M, et al. Persistent autoantibody-production by intermediates between short-and long-lived plasma cells in inflamed lymph nodes of experimental epidermolysis bullosa acquisita. *PLoS One*. 2013;8(12):e83631.
63. Diebold J, Rüdiger T, Marx A, Müller-Hermelink HK. Funktionelle Anatomie, allgemeine Pathologie und Mitbeteiligung der Milz bei Erkrankungen anderer Organe. In: Müller-Hermelink HK, Kreipe HH, editors. *Pathologie: Knochenmark, Lymphatisches System, Milz, Thymus*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2019. p. 237-97.
64. William J, Euler C, Shlomchik MJ. Short-lived plasmablasts dominate the early spontaneous rheumatoid factor response: differentiation pathways, hypermutating cell types, and affinity maturation outside the germinal center. *J Immunol*. 2005;174(11):6879-87.
65. Voss LF, Howarth AJ, Wittenborn TR, Hummelgaard S, Juul-Madsen K, Kastberg KS, et al. The extrafollicular response is sufficient to drive initiation of autoimmunity and early disease hallmarks of lupus. *Front Immunol*. 2022;13:1021370.
66. Gonzalez-Garcia I, Ocana E, Jimenez-Gomez G, Campos-Caro A, Brieva JA. Immunization-induced perturbation of human blood plasma cell pool: progressive maturation, IL-6 responsiveness, and high PRDI-BF1/BLIMP1 expression are critical distinctions between antigen-specific and nonspecific plasma cells. *J Immunol*. 2006;176(7):4042-50.
67. Minges Wols HA, Underhill GH, Kansas GS, Witte PL. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *J Immunol*. 2002;169(8):4213-21.
68. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (T<sub>fh</sub>) differentiation. *PLoS One*. 2011;6(3):e17739.
69. Zografou C, Vakrakou AG, Stathopoulos P. Short- and Long-Lived Autoantibody-Secreting Cells in Autoimmune Neurological Disorders. *Front Immunol*. 2021;12:686466.
70. Toellner KM, Khan M, Sze DM. Analysis of the germinal center reaction and in vivo long-lived plasma cells. *Methods Mol Biol*. 2004;271:111-25.
71. Corcoran LM, Tarlinton DM. Regulation of germinal center responses, memory B cells and plasma cell formation-an update. *Curr Opin Immunol*. 2016;39:59-67.

72. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, et al. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol.* 2003;171(4):1684-90.
73. Manz RA, Radbruch A. Plasma cells for a lifetime? *Eur J Immunol.* 2002;32(4):923-7.
74. Wilmore JR, Gaudette BT, Gomez Atria D, Rosenthal RL, Reiser SK, Meng W, et al. IgA Plasma Cells Are Long-Lived Residents of Gut and Bone Marrow That Express Isotype- and Tissue-Specific Gene Expression Patterns. *Front Immunol.* 2021;12:791095.
75. Cassese G, Lindenau S, de Boer B, Arce S, Hauser A, Riemekasten G, et al. Inflamed kidneys of NZB / W mice are a major site for the homeostasis of plasma cells. *Eur J Immunol.* 2001;31(9):2726-32.
76. Sze DM, Toellner KM, Garcia de Vinuesa C, Taylor DR, MacLennan IC. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *J Exp Med.* 2000;192(6):813-21.
77. Slifka MK, Matloubian M, Ahmed R. Bone-Marrow Is a Major Site of Long-Term Antibody-Production after Acute Viral-Infection. *J Virol.* 1995;69(3):1895-902.
78. Ellyard JI, Avery DT, Phan TG, Hare NJ, Hodgkin PD, Tangye SG. Antigen-selected, immunoglobulin-secreting cells persist in human spleen and bone marrow. *Blood.* 2004;103(10):3805-12.
79. Hensvold A, Horuluoglu B, Sahlstrom P, Thyagarajan R, Diaz Boada JS, Hansson M, et al. The human bone marrow plasma cell compartment in rheumatoid arthritis - Clonal relationships and anti-citrulline autoantibody producing cells. *J Autoimmun.* 2023;136:103022.
80. Mei HE, Yoshida T, Sime W, Hiepe F, Thiele K, Manz RA, et al. Blood-borne human plasma cells in steady state are derived from mucosal immune responses. *Blood.* 2009;113(11):2461-9.
81. Bohannon C, Powers R, Satyabhama L, Cui A, Tipton C, Michaeli M, et al. Long-lived antigen-induced IgM plasma cells demonstrate somatic mutations and contribute to long-term protection. *Nature Communications.* 2016;7.
82. Brandtzaeg P, Johansen FE. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunol Rev.* 2005;206:32-63.
83. Mesin L, Di Niro R, Thompson KM, Lundin KE, Sollid LM. Long-lived plasma cells from human small intestine biopsies secrete immunoglobulins for many weeks in vitro. *J Immunol.* 2011;187(6):2867-74.
84. Ahuja A, Anderson SM, Khalil A, Shlomchik MJ. Maintenance of the plasma cell pool is independent of memory B cells. *Proc Natl Acad Sci U S A.* 2008;105(12):4802-7.
85. DiLillo DJ, Hamaguchi Y, Ueda Y, Yang K, Uchida J, Haas KM, et al. Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. *J Immunol.* 2008;180(1):361-71.
86. Liu YJ, Barthelemy C, de Bouteiller O, Arpin C, Durand I, Banchereau J. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity.* 1995;2(3):239-48.
87. Budeus B, Kibler A, Kuppers R. Human IgM-expressing memory B cells. *Front Immunol.* 2023;14:1308378.
88. Ciocca M, Zaffina S, Fernandez Salinas A, Bocci C, Palomba P, Conti MG, et al. Evolution of Human Memory B Cells From Childhood to Old Age. *Front Immunol.* 2021;12:690534.
89. Paramithiotis E, Cooper MD. Memory B lymphocytes migrate to bone marrow in humans. *Proc Natl Acad Sci U S A.* 1997;94(1):208-12.

90. Mamani-Matsuda M, Cosma A, Weller S, Faili A, Staib C, Garçon L, et al. The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells. *Blood*. 2008;111(9):4653-9.
91. Benson MJ, Dillon SR, Castigli E, Geha RS, Xu S, Lam KP, et al. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. *J Immunol*. 2008;180(6):3655-9.
92. Darce JR, Arendt BK, Chang SK, Jelinek DF. Divergent effects of BAFF on human memory B cell differentiation into Ig-secreting cells. *J Immunol*. 2007;178(9):5612-22.
93. Ng LG, Sutherland AP, Newton R, Qian F, Cachero TG, Scott ML, et al. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. *J Immunol*. 2004;173(2):807-17.
94. Smulski CR, Eibel H. BAFF and BAFF-Receptor in B Cell Selection and Survival. *Front Immunol*. 2018;9:2285.
95. Muller-Winkler J, Mitter R, Rappe JCF, Vanes L, Schweighoffer E, Mohammadi H, et al. Critical requirement for BCR, BAFF, and BAFFR in memory B cell survival. *J Exp Med*. 2021;218(2).
96. Lau AWY, Turner VM, Bourne K, Hermes JR, Chan TD, Brink R. BAFFR controls early memory B cell responses but is dispensable for germinal center function. *J Exp Med*. 2021;218(2).
97. Brooks JF, Zikherman J. The BAFFling persistence of memory B cells. *J Exp Med*. 2021;218(2).
98. Smith KG, Weiss U, Rajewsky K, Nossal GJ, Tarlinton DM. Bcl-2 increases memory B cell recruitment but does not perturb selection in germinal centers. *Immunity*. 1994;1(9):803-13.
99. Suan D, Sundling C, Brink R. Plasma cell and memory B cell differentiation from the germinal center. *Curr Opin Immunol*. 2017;45:97-102.
100. Good KL, Avery DT, Tangye SG. Resting Human Memory B Cells Are Intrinsically Programmed for Enhanced Survival and Responsiveness to Diverse Stimuli Compared to Naive B Cells. *Journal of Immunology*. 2009;182(2):890-901.
101. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting Edge: Long-Term B Cell Memory in Humans after Smallpox Vaccination *The Journal of Immunology*. 2003;171(10):4969-73.
102. Moran I, Nguyen A, Khoo WH, Butt D, Bourne K, Young C, et al. Memory B cells are reactivated in subcapsular proliferative foci of lymph nodes. *Nat Commun*. 2018;9(1):3372.
103. Palm AE, Henry C. Remembrance of Things Past: Long-Term B Cell Memory After Infection and Vaccination. *Front Immunol*. 2019;10:1787.
104. Rottach E-M. Generierung und Charakterisierung neuer Marker für Hühner B-Zellen. Germany: Ludwig Maximilians Universität; 2012.
105. Goodnow CC, Crosbie J, Jorgensen H, Brink RA, Basten A. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature*. 1989;342(6248):385-91.
106. Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*. 2003;426(6967):671-6.
107. Vikstrom I, Carotta S, Luthje K, Peperzak V, Jost PJ, Glaser S, et al. Mcl-1 is essential for germinal center formation and B cell memory. *Science*. 2010;330(6007):1095-9.

108. MacLennan IC, Gulbranson-Judge A, Toellner KM, Casamayor-Palleja M, Chan E, Sze DM, et al. The changing preference of T and B cells for partners as T-dependent antibody responses develop. *Immunol Rev.* 1997;156:53-66.
109. Wehrli N, Legler DF, Finke D, Toellner KM, Loetscher P, Baggiolini M, et al. Changing responsiveness to chemokines allows medullary plasmablasts to leave lymph nodes. *Eur J Immunol.* 2001;31(2):609-16.
110. Ho F, Lortan JE, MacLennan IC, Khan M. Distinct short-lived and long-lived antibody-producing cell populations. *Eur J Immunol.* 1986;16(10):1297-301.
111. MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zuniga E, et al. Extrafollicular antibody responses. *Immunol Rev.* 2003;194:8-18.
112. Moser K, Tokoyoda K, Radbruch A, MacLennan I, Manz RA. Stromal niches, plasma cell differentiation and survival. *Curr Opin Immunol.* 2006;18(3):265-70.
113. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dorner T, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat Rev Immunol.* 2006;6(10):741-50.
114. Fecteau JF, Cote G, Neron S. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. *J Immunol.* 2006;177(6):3728-36.
115. Blanco E, Perez-Andres M, Arriba-Mendez S, Contreras-Sanfeliciano T, Criado I, Pelak O, et al. Age-associated distribution of normal B-cell and plasma cell subsets in peripheral blood. *J Allergy Clin Immunol.* 2018;141(6):2208-19 e16.
116. Adams B, Dorfler P, Aguzzi A, Kozmik Z, Urbanek P, Maurer-Fogy I, et al. Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev.* 1992;6(9):1589-607.
117. Nutt SL, Morrison AM, Dorfler P, Rolink A, Busslinger M. Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *EMBO J.* 1998;17(8):2319-33.
118. O'Brien P, Morin P, Jr., Ouellette RJ, Robichaud GA. The Pax-5 gene: a pluripotent regulator of B-cell differentiation and cancer disease. *Cancer Res.* 2011;71(24):7345-50.
119. Bullerwell CE, Robichaud PP, Deprez PML, Joy AP, Wajnberg G, D'Souza D, et al. EBF1 drives hallmark B cell gene expression by enabling the interaction of PAX5 with the MLL H3K4 methyltransferase complex. *Sci Rep.* 2021;11(1):1537.
120. Shahjahani M, Norozi F, Ahmadzadeh A, Shahrabi S, Tavakoli F, Asnafi AA, et al. The role of Pax5 in leukemia: diagnosis and prognosis significance. *Med Oncol.* 2015;32(1):360.
121. Chung EY, Psathas JN, Yu D, Li Y, Weiss MJ, Thomas-Tikhonenko A. CD19 is a major B cell receptor-independent activator of MYC-driven B-lymphomagenesis. *J Clin Invest.* 2012;122(6):2257-66.
122. Chang CC, Ye BH, Chaganti RS, Dalla-Favera R. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. *Proc Natl Acad Sci U S A.* 1996;93(14):6947-52.
123. Shaffer AL, Yu X, He Y, Boldrick J, Chan EP, Staudt LM. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity.* 2000;13(2):199-212.
124. Ranuncolo SM, Polo JM, Dierov J, Singer M, Kuo T, Grealley J, et al. Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nat Immunol.* 2007;8(7):705-14.



125. Duy C, Yu JJ, Nahar R, Swaminathan S, Kweon SM, Polo JM, et al. BCL6 is critical for the development of a diverse primary B cell repertoire. *J Exp Med*. 2010;207(6):1209-21.
126. Fairfax KA, Corcoran LM, Pridans C, Huntington ND, Kallies A, Nutt SL, et al. Different kinetics of blimp-1 induction in B cell subsets revealed by reporter gene. *J Immunol*. 2007;178(7):4104-11.
127. Schmidlin H, Diehl SA, Nagasawa M, Scheeren FA, Schotte R, Uittenbogaart CH, et al. Spi-B inhibits human plasma cell differentiation by repressing BLIMP1 and XBP-1 expression. *Blood*. 2008;112(5):1804-12.
128. Yasuda T, Hayakawa F, Kurahashi S, Sugimoto K, Minami Y, Tomita A, et al. B cell receptor-ERK1/2 signal cancels PAX5-dependent repression of BLIMP1 through PAX5 phosphorylation: a mechanism of antigen-triggering plasma cell differentiation. *J Immunol*. 2012;188(12):6127-34.
129. Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity*. 2002;17(1):51-62.
130. Nadeau S, Martins GA. Conserved and Unique Functions of Blimp1 in Immune Cells. *Front Immunol*. 2021;12:805260.
131. Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol*. 2004;173(9):5361-71.
132. Moore PA, Belvedere O, Orr A, Pieri K, LaFleur DW, Feng P, et al. BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science*. 1999;285(5425):260-3.
133. Hsu BL, Harless SM, Lindsley RC, Hilbert DM, Cancro MP. Cutting edge: BLyS enables survival of transitional and mature B cells through distinct mediators. *J Immunol*. 2002;168(12):5993-6.
134. Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, et al. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med*. 1999;189(11):1747-56.
135. Gross JA, Dillon SR, Mudri S, Johnston J, Littau A, Roque R, et al. TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. impaired B cell maturation in mice lacking BLyS. *Immunity*. 2001;15(2):289-302.
136. Bossen C, Cachero TG, Tardivel A, Ingold K, Willen L, Dobles M, et al. TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. *Blood*. 2008;111(3):1004-12.
137. Bossen C, Schneider P. BAFF, APRIL and their receptors: structure, function and signaling. *Semin Immunol*. 2006;18(5):263-75.
138. Cornelis R, Chang HD, Radbruch A. Keeping up with the stress of antibody production: BAFF and APRIL maintain memory plasma cells. *Curr Opin Immunol*. 2021;71:97-102.
139. Ingold K, Zumsteg A, Tardivel A, Huard B, Steiner QG, Cachero TG, et al. Identification of proteoglycans as the APRIL-specific binding partners. *J Exp Med*. 2005;201(9):1375-83.
140. Lightman SM, Utey A, Lee KP. Survival of Long-Lived Plasma Cells (LLPC): Piecing Together the Puzzle. *Front Immunol*. 2019;10:965.
141. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol*. 2002;3(9):822-9.

142. He B, Xu W, Santini PA, Polydorides AD, Chiu A, Estrella J, et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity*. 2007;26(6):812-26.
143. Mhaweche-Fauceglia P, Kaya G, Sauter G, McKee T, Donze O, Schwaller J, et al. The source of APRIL up-regulation in human solid tumor lesions. *J Leukoc Biol*. 2006;80(4):697-704.
144. Polverino F, Laucho-Contreras M, Rojas Quintero J, Divo M, Pinto-Plata V, Sholl L, et al. Increased expression of A Proliferation-inducing Ligand (APRIL) in lung leukocytes and alveolar epithelial cells in COPD patients with non small cell lung cancer: a possible link between COPD and lung cancer? *Multidiscip Respir Med*. 2016;11:17.
145. Bishop GA, Hostager BS. Signaling by CD40 and its mimics in B cell activation. *Immunol Res*. 2001;24(2):97-109.
146. Bishop GA, Hostager BS. The CD40-CD154 interaction in B cell-T cell liaisons. *Cytokine Growth Factor Rev*. 2003;14(3-4):297-309.
147. Carrasco YR, Fleire SJ, Cameron T, Dustin ML, Batista FD. LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. *Immunity*. 2004;20(5):589-99.
148. Collins M, Ling V, Carreno BM. The B7 family of immune-regulatory ligands. *Genome Biol*. 2005;6(6):223.
149. Clatza A, Bonifaz LC, Vignali DA, Moreno J. CD40-induced aggregation of MHC class II and CD80 on the cell surface leads to an early enhancement in antigen presentation. *J Immunol*. 2003;171(12):6478-87.
150. Armitage RJ, Macduff BM, Spriggs MK, Fanslow WC. Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. *J Immunol*. 1993;150(9):3671-80.
151. Arpin C, Dechanet J, Van Kooten C, Merville P, Grouard G, Briere F, et al. Generation of memory B cells and plasma cells in vitro. *Science*. 1995;268(5211):720-2.
152. Delfraissy JF, Wallon C, Galanaud P. Interferon-alpha can synergize with interleukin 2 for human in vitro antibody response. *Eur J Immunol*. 1988;18(9):1379-84.
153. Kehrl JH, Muraguchi A, Fauci AS. The Effects of Interleukin-1, Interleukin-2, Alpha-Interferon, and Gamma-Interferon on Human Lymphocytes-B. *T Assoc Am Physician*. 1984;97:182-9.
154. Mingari MC, Gerosa F, Carra G, Accolla RS, Moretta A, Zubler RH, et al. Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells. *Nature*. 1984;312(5995):641-3.
155. Nakagawa T, Hirano T, Nakagawa N, Yoshizaki K, Kishimoto T. Effect of Recombinant IL-2 and Gamma-Ifn on Proliferation and Differentiation of Human B-Cells. *Journal of Immunology*. 1985;134(2):959-66.
156. Liao W, Lin JX, Leonard WJ. Interleukin-2 at the Crossroads of Effector Responses, Tolerance, and Immunotherapy. *Immunity*. 2013;38(1):13-25.
157. Evans SS, Repasky EA, Fisher DT. Fever and the thermal regulation of immunity: the immune system feels the heat. *Nat Rev Immunol*. 2015;15(6):335-49.
158. Splawski JB, McAnally LM, Lipsky PE. IL-2 dependence of the promotion of human B cell differentiation by IL-6 (BSF-2). *J Immunol*. 1990;144(2):562-9.

159. Dienz O, Eaton SM, Bond JP, Neveu W, Moquin D, Noubade R, et al. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. *J Exp Med*. 2009;206(1):69-78.
160. Saeland S, Duvert V, Moreau I, Banchereau J. Human B-Cell Precursors Proliferate and Express CD23 after CD40 Ligation. *Journal of Experimental Medicine*. 1993;178(1):113-20.
161. Fluckiger AC, Garrone P, Durand I, Galizzi JP, Banchereau J. Interleukin 10 (IL-10) up-Regulates Functional High-Affinity IL-2 Receptors on Normal and Leukemic B-Lymphocytes. *Journal of Experimental Medicine*. 1993;178(5):1473-81.
162. Itoh K, Hirohata S. The role of IL-10 in human B cell activation, proliferation, and differentiation. *J Immunol*. 1995;154(9):4341-50.
163. Levy Y, Brouet JC. Interleukin-10 Prevents Spontaneous Death of Germinal Center B-Cells by Induction of the Bcl-2 Protein. *Journal of Clinical Investigation*. 1994;93(1):424-8.
164. Hu BT, Insel RA. Up-regulation of telomerase in human B lymphocytes occurs independently of cellular proliferation and with expression of the telomerase catalytic subunit. *Eur J Immunol*. 1999;29(11):3745-53.
165. Rousset F, Garcia E, Defrance T, Peronne C, Vezzio N, Hsu DH, et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A*. 1992;89(5):1890-3.
166. Defrance T, Vanbervliet B, Briere F, Durand I, Rousset F, Banchereau J. Interleukin-10 and Transforming Growth-Factor-Beta Cooperate to Induce Anti-Cd40 Activated Naive Human B-Cells to Secrete Immunoglobulin-A. *Journal of Experimental Medicine*. 1992;175(3):671-82.
167. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol*. 2007;179(12):8180-90.
168. Good KL, Bryant VL, Tangye SG. Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21. *J Immunol*. 2006;177(8):5236-47.
169. Avery DT, Deenick EK, Ma CS, Suryani S, Simpson N, Chew GY, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J Exp Med*. 2010;207(1):155-71.
170. Diehl SA, Schmidlin H, Nagasawa M, van Haren SD, Kwakkenbos MJ, Yasuda E, et al. STAT3-mediated up-regulation of BLIMP1 is coordinated with BCL6 down-regulation to control human plasma cell differentiation. *Journal of Immunology*. 2008;180(7):4805-15.
171. Konforte D, Simard N, Paige CJ. IL-21: an executor of B cell fate. *J Immunol*. 2009;182(4):1781-7.
172. Avery DT, Bryant VL, Ma CS, Malefyt RD, Tangye SG. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *Journal of Immunology*. 2008;181(3):1767-79.
173. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol*. 2005;175(12):7867-79.
174. Pene J, Guglielmi L, Gauchat JF, Harrer N, Woisetschlager M, Boulay V, et al. IFN-gamma-mediated inhibition of human IgE synthesis by IL-21 is associated with a polymorphism in the IL-21R gene. *J Immunol*. 2006;177(8):5006-13.
175. Pene J, Gauchat JF, Lecart S, Drouet E, Guglielmi P, Boulay V, et al. Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. *J Immunol*. 2004;172(9):5154-7.

176. Kuchen S, Robbins R, Sims GP, Sheng C, Phillips TM, Lipsky PE, et al. Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4 T cell-B cell collaboration. *Journal of Immunology*. 2007;179(9):5886-96.
177. Yoon SO, Zhang X, Berner P, Choi YS. IL-21 and IL-10 have redundant roles but differential capacities at different stages of Plasma Cell generation from human Germinal Center B cells. *J Leukoc Biol*. 2009;86(6):1311-8.
178. Borsutzky S, Cazac BB, Roes J, Guzmán CA. TGF- $\beta$  receptor signaling is critical for mucosal IgA responses. *Journal of Immunology*. 2004;173(5):3305-9.
179. Stavnezer J, Kang J. The Surprising Discovery That TGF $\beta$  Specifically Induces the IgA Class Switch. *Journal of Immunology*. 2009;182(1):5-7.
180. Jurado A, Carballido J, Griffel H, Hochkeppel HK, Wetzel GD. The immunomodulatory effects of interferon-gamma on mature B-lymphocyte responses. *Experientia*. 1989;45(6):521-6.
181. Snapper CM, McIntyre TM, Mandler R, Pecanha LM, Finkelman FD, Lees A, et al. Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J Exp Med*. 1992;175(5):1367-71.
182. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. *Blood*. 2008;112(5):1570-80.
183. Perez-Andres M, Paiva B, Nieto WG, Caraux A, Schmitz A, Almeida J, et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. *Cytometry B Clin Cytom*. 2010;78 Suppl 1:S47-60.
184. Hoffman W, Lakkis FG, Chalasani G. B Cells, Antibodies, and More. *Clin J Am Soc Nephrol*. 2016;11(1):137-54.
185. Kurosaki T, Kometani K, Ise W. Memory B cells. *Nat Rev Immunol*. 2015;15(3):149-59.
186. Liechti T, Roederer M. OMIP-051 - 28-color flow cytometry panel to characterize B cells and myeloid cells. *Cytometry A*. 2019;95(2):150-5.
187. Sarvaria A, Madrigal JA, Saudemont A. B cell regulation in cancer and anti-tumor immunity. *Cell Mol Immunol*. 2017;14(8):662-74.
188. Naradikian MS, Scholz JL, Oropallo MA, Cancro MP. Understanding B Cell Biology. In: Bosch X, Ramos-Casals M, Khamashta MA, editors. *Drugs Targeting B-Cells in Autoimmune Diseases*. Basel: Springer Basel; 2014. p. 11-35.
189. Shen P, Fillatreau S. Antibody-independent functions of B cells: a focus on cytokines. *Nat Rev Immunol*. 2015;15(7):441-51.
190. Allman D, Pillai S. Peripheral B cell subsets. *Curr Opin Immunol*. 2008;20(2):149-57.
191. Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. *Immunol Rev*. 2004;197:179-91.
192. Kurosaki T. B-lymphocyte biology. *Immunol Rev*. 2010;237(1):5-9.
193. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. *J Allergy Clin Immunol*. 2013;131(4):959-71.
194. Weill JC, Weller S, Reynaud CA. Human marginal zone B cells. *Annu Rev Immunol*. 2009;27:267-85.
195. Khodadadi L, Cheng Q, Radbruch A, Hiepe F. The Maintenance of Memory Plasma Cells. *Front Immunol*. 2019;10:721.

196. Sanz I, Wei C, Jenks SA, Cashman KS, Tipton C, Woodruff MC, et al. Challenges and Opportunities for Consistent Classification of Human B Cell and Plasma Cell Populations. *Front Immunol.* 2019;10:2458.
197. Garcia-Carmona Y, Fribourg M, Sowa A, Cerutti A, Cunningham-Rundles C. TACI and endogenous APRIL in B cell maturation. *Clin Immunol.* 2023;253:109689.
198. Garcia-Carmona Y, Cols M, Ting AT, Radigan L, Yuk FJ, Zhang L, et al. Differential induction of plasma cells by isoforms of human TACI. *Blood.* 2015;125(11):1749-58.
199. Brynjolfsson SF, Persson Berg L, Olsen Ekerhult T, Rimkute I, Wick MJ, Martensson IL, et al. Long-Lived Plasma Cells in Mice and Men. *Front Immunol.* 2018;9:2673.
200. Delaloy C, Schuh W, Jack HM, Bonaud A, Espeli M. Single-cell resolution of plasma cell fate programming in health and disease. *Eur J Immunol.* 2022;52(1):10-23.
201. Jackson SM, Harp N, Patel D, Zhang J, Willson S, Kim YJ, et al. CD45RO enriches for activated, highly mutated human germinal center B cells. *Blood.* 2007;110(12):3917-25.
202. Adelman HB. The embryological treatises of hieronymus fabricius of aquapendente. The formation of the egg and of the chick [De Formatione Ovi et Pulli]: The formed fetus [De Formato Foetu]. A facsimile edition, with an introduction, a translation, and a commentary by Howard B. Adelman. Ithaca, NY, USA: Cornell University Press; 1942. 833 p.
203. Taylor RL, Jr., McCorkle FM, Jr. A landmark contribution to poultry science-- Immunological function of the bursa of Fabricius. *Poult Sci.* 2009;88(4):816-23.
204. Weber WT, Foglia LM. Evidence for the Presence of Precursor B-Cells in Normal and in Hormonally Bursectomized Chick-Embryos. *Cellular Immunology.* 1980;52(1):84-94.
205. Mansikka A, Sandberg M, Lassila O, Toivanen P. Rearrangement of Immunoglobulin Light Chain Genes in the Chicken Occurs Prior to Colonization of the Embryonic Bursa of Fabricius. *P Natl Acad Sci USA.* 1990;87(23):9416-20.
206. Pink JR, Vainio O, Rijnbeek AM. Clones of Lymphocytes-B in Individual Follicles of the Bursa of Fabricius. *European Journal of Immunology.* 1985;15(1):83-7.
207. Houssaint E, Belo M, Le Douarin NM. Investigations on cell lineage and tissue interactions in the developing bursa of Fabricius through interspecific chimeras. *Dev Biol.* 1976;53(2):250-64.
208. Nagy N, Busalt F, Halasy V, Kohn M, Schmieder S, Fejszak N, et al. In and Out of the Bursa-The Role of CXCR4 in Chicken B Cell Development. *Front Immunol.* 2020;11:1468.
209. McCormack WT, Tjoelker LW, Barth CF, Carlson LM, Petryniak B, Humphries EH, et al. Selection for B-Cells with Productive Igl Gene Rearrangements Occurs in the Bursa of Fabricius during Chicken Embryonic-Development. *Gene Dev.* 1989;3(6):838-47.
210. Bockman DE, Cooper MD. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. *Am J Anat.* 1973;136(4):455-77.
211. Ratcliffe MJ, Lassila O, Pink JR, Vainio O. Avian B cell precursors: surface immunoglobulin expression is an early, possibly bursa-independent event. *Eur J Immunol.* 1986;16(2):129-33.
212. Reynaud CA, Anquez V, Grimal H, Weill JC. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell.* 1987;48(3):379-88.
213. McCormack WT, Tjoelker LW, Thompson CB. Avian B-cell development: generation of an immunoglobulin repertoire by gene conversion. *Annu Rev Immunol.* 1991;9:219-41.

214. Reynaud CA, Bertocci B, Dahan A, Weill JC. Formation of the chicken B-cell repertoire: ontogenesis, regulation of Ig gene rearrangement, and diversification by gene conversion. *Adv Immunol.* 1994;57:353-78.
215. Cooper MD, Cain WA, Van Alten PJ, Good RA. Development and function of the immunoglobulin producing system. I. Effect of bursectomy at different stages of development on germinal centers, plasma cells, immunoglobulins and antibody production. *Int Arch Allergy Appl Immunol.* 1969;35(3):242-52.
216. Cain WA, Cooper MD, Van Alten PJ, Good RA. Development and function of the immunoglobulin-producing system. II. Role of the bursa in the development of humoral immunological competence. *J Immunol.* 1969;102(3):671-8.
217. Paramithiotis E, Ratcliffe MJ. B cell emigration directly from the cortex of lymphoid follicles in the bursa of Fabricius. *Eur J Immunol.* 1994;24(2):458-63.
218. Paramithiotis E, Ratcliffe MJ. Bursa-dependent subpopulations of peripheral B lymphocytes in chicken blood. *Eur J Immunol.* 1993;23(1):96-102.
219. Motyka B, Reynolds JD. Apoptosis is associated with the extensive B cell death in the sheep ileal Peyer's patch and the chicken bursa of Fabricius: a possible role in B cell selection. *Eur J Immunol.* 1991;21(8):1951-8.
220. Lassila O. Emigration of B cells from chicken bursa of Fabricius. *Eur J Immunol.* 1989;19(5):955-8.
221. Bellamy D, Mohamed K. A comparative study of age involution of the bursa of Fabricius and thymus in birds. *Thymus.* 1982;4(2):107-14.
222. Chen KL, Tsay SM, Chiou PW, Sun CP, Weng BC. Effects of caponization and different forms of exogenous androgen implantation on immunity in male chicks. *Poult Sci.* 2010;89(5):887-94.
223. Paramithiotis E, Ratcliffe MJ. Survivors of bursal B cell production and emigration. *Poult Sci.* 1994;73(7):991-7.
224. Toivanen P, Toivanen A. Bursal and postbursal stem cells in chicken. Functional characteristics. *Eur J Immunol.* 1973;3(9):585-95.
225. Yasuda M, Taura Y, Yokomizo Y, Ekino S. A comparative study of germinal center: fowls and mammals. *Comp Immunol Microbiol Infect Dis.* 1998;21(3):179-89.
226. Arakawa H, Furusawa S, Ekino S, Yamagishi H. Immunoglobulin gene hyperconversion ongoing in chicken splenic germinal centers. *EMBO J.* 1996;15(10):2540-6.
227. Arakawa H, Kuma K, Yasuda M, Furusawa S, Ekino S, Yamagishi H. Oligoclonal development of B cells bearing discrete Ig chains in chicken single germinal centers. *J Immunol.* 1998;160(9):4232-41.
228. Kohonen P, Nera KP, Lassila O. Avian model for B-cell immunology--new genomes and phylotranscriptomics. *Scand J Immunol.* 2007;66(2-3):113-21.
229. Reynaud CA, Imhof BA, Anquez V, Weill JC. Emergence of committed B lymphoid progenitors in the developing chicken embryo. *EMBO J.* 1992;11(12):4349-58.
230. Le Douarin NM, Houssaint E, Jotereau FV, Belo M. Origin of hemopoietic stem cells in embryonic bursa of Fabricius and bone marrow studied through interspecific chimeras. *Proc Natl Acad Sci U S A.* 1975;72(7):2701-5.
231. Houssaint E, Mansikka A, Vainio O. Early separation of B and T lymphocyte precursors in chick embryo. *J Exp Med.* 1991;174(2):397-406.



232. Benatar T, Tkalec L, Ratcliffe MJ. Stochastic rearrangement of immunoglobulin variable-region genes in chicken B-cell development. *Proc Natl Acad Sci U S A*. 1992;89(16):7615-9.
233. Thompson CB, Neiman PE. Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell*. 1987;48(3):369-78.
234. Lydyard PM, Grossi CE, Cooper MD. Ontogeny of B cells in the chicken. I. Sequential development of clonal diversity in the bursa. *J Exp Med*. 1976;144(1):79-97.
235. Reynolds JD. Mitotic rate maturation in the Peyer's patches of fetal sheep and in the bursa of Fabricius of the chick embryo. *Eur J Immunol*. 1987;17(4):503-7.
236. Jeurissen SH. The role of various compartments in the chicken spleen during an antigen-specific humoral response. *Immunology*. 1993;80(1):29-33.
237. Nagy N, Olah I, Vervelde L. Structure of the avian lymphoid system. In: Kaspers B, Schat KA, Göbel TW, Vervelde L, editors. *Avian Immunology*. 3 ed: Academic Press, Elsevier Ltd.; 2022. p. 11-44.
238. Scott TR, Savage ML, Olah I. Plasma cells of the chicken Harderian gland. *Poult Sci*. 1993;72(7):1273-9.
239. Jeurissen SH, Janse EM, Koch G, De Boer GF. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res*. 1989;258(1):119-24.
240. Houssaint E, Diez E, Pink JR. Ontogeny and tissue distribution of the chicken Bu-1a antigen. *Immunology*. 1987;62(3):463-70.
241. Nagase F, Nakashima I, Kato N. Studies on the immune response in chickens. IV. Generation of hapten-specific memory and absence of increase in carrier-specific helper memory in antibody response to sheep red blood cell antigen and its hapten-conjugate. *Dev Comp Immunol*. 1983;7(1):127-37.
242. Kothlow S, Morgenroth I, Tregaskes CA, Kaspers B, Young JR. CD40 ligand supports the long-term maintenance and differentiation of chicken B cells in culture. *Dev Comp Immunol*. 2008;32(9):1015-26.
243. Schneider K, Kothlow S, Schneider P, Tardivel A, Gobel T, Kaspers B, et al. Chicken BAFF--a highly conserved cytokine that mediates B cell survival. *Int Immunol*. 2004;16(1):139-48.
244. Kothlow S, Schenk-Weibhauser K, Ratcliffe MJ, Kaspers B. Prolonged effect of BAFF on chicken B cell development revealed by RCAS retroviral gene transfer in vivo. *Mol Immunol*. 2010;47(7-8):1619-28.
245. Kothlow S, Morgenroth I, Graef Y, Schneider K, Riehl I, Staeheli P, et al. Unique and conserved functions of B cell-activating factor of the TNF family (BAFF) in the chicken. *Int Immunol*. 2007;19(2):203-15.
246. van Haarlem DA, van Kooten PJ, Rothwell L, Kaiser P, Vervelde L. Characterisation and expression analysis of the chicken interleukin-7 receptor alpha chain. *Dev Comp Immunol*. 2009;33(9):1018-26.
247. Huo S, Wang L, Zhang Y, Zhang J, Zuo Y, Xu J, et al. Molecular cloning of chicken IL-7 and characterization of its antiviral activity against IBDV in vivo. *Poult Sci*. 2016;95(11):2647-54.
248. Wu Z, Hu T, Rothwell L, Vervelde L, Kaiser P, Boulton K, et al. Analysis of the function of IL-10 in chickens using specific neutralising antibodies and a sensitive capture ELISA. *Dev Comp Immunol*. 2016;63:206-12.
249. Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, et al. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. *J Immunol*. 2004;173(4):2675-82.

250. Rothwell L, Hu TJ, Wu ZG, Kaiser P. Chicken interleukin-21 is costimulatory for T cells and blocks maturation of dendritic cells. *Developmental and Comparative Immunology*. 2012;36(2):475-82.
251. Koskela K, Arstila TP, Lassila O. Costimulatory function of CD28 in avian gammadelta T cells is evolutionarily conserved. *Scand J Immunol*. 1998;48(6):635-41.
252. Chu VT, Enghard P, Schurer S, Steinhauser G, Rudolph B, Riemekasten G, et al. Systemic activation of the immune system induces aberrant BAFF and APRIL expression in B cells in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2009;60(7):2083-93.
253. Reddy SK, Hu T, Gudivada R, Staines KA, Wright KE, Vickerstaff L, et al. The BAFF-Interacting receptors of chickens. *Dev Comp Immunol*. 2008;32(9):1076-87.
254. Kaiser P, Poh TY, Rothwell L, Avery S, Balu S, Pathania US, et al. A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res*. 2005;25(8):467-84.
255. Tregaskes CA, Glansbeek HL, Gill AC, Hunt LG, Burnside J, Young JR. Conservation of biological properties of the CD40 ligand, CD154 in a non-mammalian vertebrate. *Dev Comp Immunol*. 2005;29(4):361-74.
256. Hartle S, Sutton K, Vervelde L, Dalgaard TS. Delineation of chicken immune markers in the era of omics and multicolor flow cytometry. *Front Vet Sci*. 2024;11:1385400.
257. Dora D, Fejszak N, Goldstein AM, Minko K, Nagy N. Ontogeny of ramified CD45 cells in chicken embryo and their contribution to bursal secretory dendritic cells. *Cell Tissue Res*. 2017;368(2):353-70.
258. Haertle S, Alzuheir I, Busalt F, Waters V, Kaiser P, Kaufer BB. Identification of the Receptor and Cellular Ortholog of the Marek's Disease Virus (MDV) CXCL Chemokine. *Front Microbiol*. 2017;8:2543.
259. Masteller EL, Larsen RD, Carlson LM, Pickel JM, Nickoloff B, Lowe J, et al. Chicken B cells undergo discrete developmental changes in surface carbohydrate structure that appear to play a role in directing lymphocyte migration during embryogenesis. *Development*. 1995;121(6):1657-67.
260. Masteller EL, Lee KP, Carlson LM, Thompson CB. Expression of sialyl Lewis(x) and Lewis(x) defines distinct stages of chicken B cell maturation. *J Immunol*. 1995;155(12):5550-6.
261. Koskinen R, Gobel TW, Tregaskes CA, Young JR, Vainio O. The structure of avian CD5 implies a conserved function. *J Immunol*. 1998;160(10):4943-50.
262. Mast J, Goddeeris BM. CD57, a marker for B-cell activation and splenic ellipsoid-associated reticular cells of the chicken. *Cell Tissue Res*. 1998;291(1):107-15.
263. Maity PC, Blount A, Jumaa H, Ronneberger O, Lillemeier BF, Reth M. B cell antigen receptors of the IgM and IgD classes are clustered in different protein islands that are altered during B cell activation. *Sci Signal*. 2015;8(394):ra93.
264. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses. *Blood*. 2009;113(16):3716-25.
265. Cortes B. Multi-faceted functions of secretory IgA at mucosal surfaces. *Front Immunol*. 2013;4:185.
266. Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A*. 2004;101(7):1981-6.

267. Lycke NY, Bemark M. The role of Peyer's patches in synchronizing gut IgA responses. *Front Immunol.* 2012;3:329.
268. Knoop KA, Newberry RD. Isolated Lymphoid Follicles are Dynamic Reservoirs for the Induction of Intestinal IgA. *Front Immunol.* 2012;3:84.
269. Kerr MA. The structure and function of human IgA. *Biochem J.* 1990;271(2):285-96.
270. Joseph J. Harnessing Nasal Immunity with IgA to Prevent Respiratory Infections. *Immuno-Basel.* 2022;2(4):571-83.
271. Knop E, Knop N, Claus P. Local production of secretory IgA in the eye-associated lymphoid tissue (EALT) of the normal human ocular surface. *Invest Ophthalmol Vis Sci.* 2008;49(6):2322-9.
272. Kijlstra A. Secretory IgA responses on the human ocular surface. *Adv Exp Med Biol.* 1998;438:575-81.
273. Ushio H, Watanabe N, Kiso Y, Higuchi S, Matsuda H. Protective immunity and mast cell and eosinophil responses in mice infested with larval *Haemaphysalis longicornis* ticks. *Parasite Immunol.* 1993;15(4):209-14.
274. Karagiannis SN, Karagiannis P, Josephs DH, Saul L, Gilbert AE, Upton N, et al. Immunoglobulin E and Allergy: Antibodies in Immune Inflammation and Treatment. *Microbiol Spectr.* 2013;1(1).
275. Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. *Annu Rev Immunol.* 2005;23:367-86.
276. Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, Meijide LM, et al. Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clin Exp Immunol.* 2008;151(1):42-50.
277. Mestecky J, Russell MW, Jackson S, Brown TA. The human IgA system: a reassessment. *Clin Immunol Immunopathol.* 1986;40(1):105-14.
278. Mestecky J, McGhee JR. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv Immunol.* 1987;40:153-245.
279. Spiegelberg HL. The structure and biology of human IgD. *Immunol Rev.* 1977;37:3-24.
280. Waldmann TA. Disorders of immunoglobulin metabolism. *N Engl J Med.* 1969;281(21):1170-7.
281. Conley ME, Delacroix DL. Intravascular and mucosal immunoglobulin A: two separate but related systems of immune defense? *Ann Intern Med.* 1987;106(6):892-9.
282. Rifai A, Fadden K, Morrison SL, Chintalacharuvu KR. The N-glycans determine the differential blood clearance and hepatic uptake of human immunoglobulin (Ig)A1 and IgA2 isotypes. *J Exp Med.* 2000;191(12):2171-82.
283. Leslie GA, Benedict AA. Structural and antigenic relationships between avian immunoglobulins. I. The immune responses of pheasants and quail and reductive dissociation of their immunoglobulins. *J Immunol.* 1969;103(6):1356-65.
284. Martins NRD, Mockett APA, Barrett ADT, Cook JKA. IgM Responses in Chicken Serum to Live and Inactivated Infectious-Bronchitis Virus-Vaccines. *Avian Diseases.* 1991;35(3):470-5.
285. Han B, Yuan H, Wang T, Li B, Ma L, Yu S, et al. Multiple IgH Isotypes Including IgD, Subclasses of IgM, and IgY Are Expressed in the Common Ancestors of Modern Birds. *J Immunol.* 2016;196(12):5138-47.

286. Parvari R, Avivi A, Lentner F, Ziv E, Tel-Or S, Burstein Y, et al. Chicken immunoglobulin gamma-heavy chains: limited VH gene repertoire, combinatorial diversification by D gene segments and evolution of the heavy chain locus. *EMBO J.* 1988;7(3):739-44.
287. Härtle S, Magor KE, Göbel TW, Davison F, Kaspers B. Chapter 5 - Structure and evolution of avian immunoglobulins. In: Kaspers B, Schat KA, Göbel TW, Vervelde L, editors. *Avian Immunology (Third Edition)*. Boston: Academic Press; 2022. p. 101-19.
288. Watanabe H, Kobayashi K. Peculiar secretory IgA system identified in chickens. *J Immunol.* 1974;113(5):1405-9.
289. Watanabe H, Kobayashi K, Isayama Y. Peculiar secretory IgA system identified in chickens. II. Identification and distribution of free secretory component and immunoglobulins of IgA, IgM, and IgG in chicken external secretions. *J Immunol.* 1975;115(4):998-1001.
290. Lebacqz-Verheyden AM, Vaerman JP, Heremans JF. Quantification and distribution of chicken immunoglobulins IgA, IgM and IgG in serum and secretions. *Immunology.* 1974;27(4):683-92.
291. Leslie GA, Clem LW. Chicken immunoglobulins: biological half-lives and normal adult serum concentrations of IgM and IgY. *Proc Soc Exp Biol Med.* 1970;134(1):195-8.
292. Leslie GA, Stankus RP, Martin LN. Secretory immunological system of fowl. V. The gallbladder: an integral part of the secretory immunological system of fowl. *Int Arch Allergy Appl Immunol.* 1976;51(2):175-85.
293. Gallego M, Glick B. The proliferative capacity of the cells of the avian Harderian gland. *Dev Comp Immunol.* 1988;12(1):157-66.
294. Yokochi T, Holly RD, Clark EA. B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. *J Immunol.* 1982;128(2):823-7.
295. Amu S, Gjertsson I, Tarkowski A, Brisslert M. B-cell CD25 expression in murine primary and secondary lymphoid tissue. *Scand J Immunol.* 2006;64(5):482-92.
296. Amu S, Tarkowski A, Dorner T, Bokarewa M, Brisslert M. The human immunomodulatory CD25+ B cell population belongs to the memory B cell pool. *Scand J Immunol.* 2007;66(1):77-86.
297. Klein U, Tu Y, Stolovitzky GA, Keller JL, Haddad J, Jr., Miljkovic V, et al. Gene expression dynamics during germinal center transit in B cells. *Ann N Y Acad Sci.* 2003;987:166-72.
298. Good-Jacobson KL, Song E, Anderson S, Sharpe AH, Shlomchik MJ. CD80 expression on B cells regulates murine T follicular helper development, germinal center B cell survival, and plasma cell generation. *J Immunol.* 2012;188(9):4217-25.
299. Darce JR, Arendt BK, Wu X, Jelinek DF. Regulated expression of BAFF-binding receptors during human B cell differentiation. *J Immunol.* 2007;179(11):7276-86.
300. Watrang E NL, Juul-Madsen HR, Dalgaard TS. Preliminary characterisation of Cd107a and Cd57 as potential activation markers of chicken cytotoxic T-cells. XII AIRG International Avian Immunology Research Group Meeting; Edinburgh, UK 2012.
301. Ramskold D, Parodis I, Lakshmikanth T, Sippl N, Khademi M, Chen Y, et al. B cell alterations during BAFF inhibition with belimumab in SLE. *EBioMedicine.* 2019;40:517-27.
302. Costes V, Magen V, Legouffe E, Durand L, Baldet P, Rossi JF, et al. The Mi15 monoclonal antibody (anti-syndecan-1) is a reliable marker for quantifying plasma cells in paraffin-embedded bone marrow biopsy specimens. *Hum Pathol.* 1999;30(12):1405-11.

303. Cheng Q, Khodadadi L, Taddeo A, Klotsche J, B FH, Radbruch A, et al. CXCR4-CXCL12 interaction is important for plasma cell homing and survival in NZB/W mice. *Eur J Immunol*. 2018;48(6):1020-9.
304. Smith KG, Hewitson TD, Nossal GJ, Tarlinton DM. The phenotype and fate of the antibody-forming cells of the splenic foci. *Eur J Immunol*. 1996;26(2):444-8.
305. Manz RA, Lohning M, Cassese G, Thiel A, Radbruch A. Survival of long-lived plasma cells is independent of antigen. *Int Immunol*. 1998;10(11):1703-11.
306. Landsverk OJ, Snir O, Casado RB, Richter L, Mold JE, Reu P, et al. Antibody-secreting plasma cells persist for decades in human intestine. *J Exp Med*. 2017;214(2):309-17.
307. Hargreaves DC, Hyman PL, Lu TT, Ngo VN, Bidgol A, Suzuki G, et al. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med*. 2001;194(1):45-56.
308. Blanc P, Moro-Sibilot L, Barthly L, Jagot F, This S, de Bernard S, et al. Mature IgM-expressing plasma cells sense antigen and develop competence for cytokine production upon antigenic challenge. *Nat Commun*. 2016;7:13600.
309. Pinto D, Montani E, Bolli M, Garavaglia G, Sallusto F, Lanzavecchia A, et al. A functional BCR in human IgA and IgM plasma cells. *Blood*. 2013;121(20):4110-4.
310. Kamata T, Nogaki F, Fagarasan S, Sakiyama T, Kobayashi I, Miyawaki S, et al. Increased frequency of surface IgA-positive plasma cells in the intestinal lamina propria and decreased IgA excretion in hyper IgA (HIGA) mice, a murine model of IgA nephropathy with hyperserum IgA. *J Immunol*. 2000;165(3):1387-94.
311. Calame KL. Plasma cells: finding new light at the end of B cell development. *Nat Immunol*. 2001;2(12):1103-8.
312. Wilkinson ST, Vanpatten KA, Fernandez DR, Brunhoeber P, Garsha KE, Glinsmann-Gibson BJ, et al. Partial plasma cell differentiation as a mechanism of lost major histocompatibility complex class II expression in diffuse large B-cell lymphoma. *Blood*. 2012;119(6):1459-67.
313. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med*. 2004;199(1):91-8.
314. Dobner M, Auerbach M, Mundt E, Icken W, Rautenschlein S. Genotype-associated differences in bursal recovery after infectious bursal disease virus (IBDV) inoculation. *Vet Immunol Immunopathol*. 2020;220:109993.
315. Rautenschlein S, Kraemer C, Vanmarcke J, Montiel E. Protective efficacy of intermediate and intermediate plus infectious bursal disease virus (IBDV) vaccines against very virulent IBDV in commercial broilers. *Avian Dis*. 2005;49(2):231-7.
316. Marsman C, Verstegen NJ, Streutker M, Jorritsma T, Boon L, Ten Brinke A, et al. Termination of CD40L co-stimulation promotes human B cell differentiation into antibody-secreting cells. *Eur J Immunol*. 2022;52(10):1662-75.
317. Possamai D, Page G, Panes R, Gagnon E, Lapointe R. CD40L-Stimulated B Lymphocytes Are Polarized toward APC Functions after Exposure to IL-4 and IL-21. *J Immunol*. 2021;207(1):77-89.
318. Quast I, Dvorscek AR, Pattaroni C, Steiner TM, McKenzie CI, Pitt C, et al. Interleukin-21, acting beyond the immunological synapse, independently controls T follicular helper and germinal center B cells. *Immunity*. 2022;55(8):1414-30 e5.

319. Sun RF, Lee J, Robinson ME, Kume K, Cosgun KN, Chan LN, et al. CD25 recruits inhibitory phosphatases for feedback control of B-cell receptor signaling. *Journal of Immunology*. 2023;210(1).
320. Brisslert M, Bokarewa M, Larsson P, Wing K, Collins LV, Tarkowski A. Phenotypic and functional characterization of human CD25+ B cells. *Immunology*. 2006;117(4):548-57.
321. Amu S, Brisslert M. Phenotype and function of CD25-expressing B lymphocytes isolated from human umbilical cord blood. *Clin Dev Immunol*. 2011;2011:481948.
322. Amu S, Gjertsson I, Brisslert M. Functional characterization of murine CD25 expressing B cells. *Scand J Immunol*. 2010;71(4):275-82.
323. Baker D, Marta M, Pryce G, Giovannoni G, Schmierer K. Memory B Cells are Major Targets for Effective Immunotherapy in Relapsing Multiple Sclerosis. *EBioMedicine*. 2017;16:41-50.
324. Kindler V, Matthes T, Jeannin P, Zubler RH. Interleukin-2 secretion by human B lymphocytes occurs as a late event and requires additional stimulation after CD40 cross-linking. *Eur J Immunol*. 1995;25(5):1239-43.
325. Toro H, Hidalgo H, Collingwood-Selby AN. Lachrymal antibody response of specific pathogen free chickens and chickens with maternal immunity after infectious bronchitis virus vaccination. *Preventive Veterinary Medicine*. 1994;18(4):267-74.
326. Toro H, Reyes E, Redmann T, Kaleta EF. Local and systemic specific antibody response of different chicken lines after ocular vaccination against infectious bronchitis. *Zentralbl Veterinarmed B*. 1996;43(8):449-54.



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