STAT6 – regulated B cell responses

STAT6 - regulierte B-Zell-Antworten

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List of Abbreviations

AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cells
BCR	B cell antigen receptor
BM	Bone marrow
CCL21	Chemokine ligand 21
CCR7	Chemokine receptor 7
CDK	Cyclin-dependent kinase
CSR	Class switch recombination
DC	Dendritic cells
FDCs	Follicular dendritic cells
FOB	Follicular B cells
GALT	Gut-associated lymphoid tissue
GC	Germinal center
IL- 4	Interleukin -4
IL-13	Interleukin- 13
ILC	Innate lymphoid cells
JAK 1	Janus Kinases 1
JAK 2	Janus Kinases 2
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharides
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MZ	Marginal Zone
Nb	Nippostrongylus brasiliensis
NK	Natural killer cells
PAMP	Pathogen-associated molecular patterns
PCs	Plasma cells
PDGF	Platelet-derived growth factor
PRR	Pattern recognition receptors
SCID	Severe combined immunodeficiency
SHM	Somatic hypermutation
SNPs	Single nucleotide polymorphisms

SRBCs	Sheep red blood cells
STAT6	Signal-transducer and activator of transcription 5
T1 B	Transitional type 1 B cells
T2 B	Transitional type 2 B cells
TAD	Terminal transactivation domain
TD	T cell-dependent
T_{FH}	Follicular T helper cells
Th2	Type 2 T helper cells
TI	T cell-independent
TLR	Toll like receptor
UTR	Untranslated region

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Zusammenfasung

Es ist allgemein bekannt, dass IL-4 an den IL-4-Typ I Rezeptor bindet und dabei die Tyrosinphosphorylierung der Januskinase (JAK) 1 und 3 induziert. Aktivierte JAKs phosphorylieren STAT6, welches dimerisiert und in den Kern von B-Zellen transloziert. Dabei wird die Expression von Zielgene reguliert, die für die Rekombination von IgE-Klassenswechsel und der keimzentrums-Bildung wichtig sind.

Unsere Gruppe hat zuvor gezeigt, dass die keimzentrums -Bildung von der STAT6-Expression in B-Zellen während der Typ-2-Immunantworten abhängt. Wir konnten ferner mehrere STAT6-regulierte Gene identifizieren, die an der B-Zell-Proliferation und dem Zellüberleben beteiligt sind. Das Ziel dieser Studie war es, neue IL-4 / IL-13-regulierte und STAT6-abhängige Gene in B-Zellen zu identifizieren, die die keimzentrums -Reaktion regulieren könnten. Daher verglichen wir die Transkriptome und Proteome von primären Maus-B-Zellen von Wildtyp- und STAT6-defizienten Mäusen, die vier Tage lang in Gegenwart oder Abwesenheit von IL-4 kultiviert wurden. Wir konnten zeigen, dass CD30, CD79b, SLP-76, DEC205, IL-5Ra, STAT5 und Thy1 in STAT6-abhängiger Weise durch IL-4 induziert werden. Andererseits wurde festgestellt, dass Syk und das Fc-Rezeptor- ähnliche Protein FcRL1 herunterreguliert sind. Diese Studie liefert die Grundlage für weitere Funktionsanalysen neu identifizierter IL-4-regulierter Proteine in B-Zellen, die möglicherweise eine Rolle bei der Bildung des Keimzentrums und der IgG1 / IgE-Umschaltung der Typ-2- Immunität spielen.

Wir bestimmten auch, wie STAT6 das Migrationsverhalten von B-Zellen in der Milz beeinflusst. Zu diesem Zweck untersuchten wir die Rolle von STAT6 für den Eintritt von B-Zellen in und die Lokalisierung innerhalb des keimzentrums anhand zweier Ansätze. Zuerst transferierten wir naive WT oder STAT6KO B-Zellen (beide CD45.2) in mit rote Blutkörperchen (vom Schaf, SRBC) immunisierten WT (CD45.1) Mäuse am Tag 8 nach der Immunisierung und analysierten deren Beitrag an der keimzentrums-Bildung am Tag 12. Hier beobachteten wir, dass nur naive WT B-Zellen zur keimzentrums-Bildung beitrugen. Zweitens verwendeten wir B-Zellen von swHEL-Mäusen, um zu untersuchen, ob alle übertragenen naiven B-Zellen von WT-Mäusen unabhängig von ihrer Antigenspezifität in das keimzentrums migrierten. Übertragene naive B-Zellen von swHEL-Mäusen befanden sich nur im Nicht- keimzentrums -Bereich des B-Zell-Follikels. Daher ziehen wir die Schlussfolgerung, dass nur Antigen-spezifische B-Zellen in keimzentrums eintreten und in diese wandern können. Die Affinitätsreifung und -differenzierung in Gedächtnis-B-Zellen oder Plasmazllen dieser „späten keimzentrums -B-Zellen“ muss noch untersucht

werden. Nachdem wir festgestellt haben, dass B-Zell-intrinsisches STAT6 für die keimzentrums - Bildung wichtig ist, untersuchten wir weiter, wie konstitutiv aktives STAT6 die B-Zell-Homöostase, keimzentrums und Plasmazlle Bildung moduliert. Dies wurde durch Charakterisierung von CD19CreSTAT6vt-Mäusen durchgeführt, die eine konstitutiv aktive Form von STAT6 (STAT6vt) nur in B-Zellen exprimieren.

Abstract

IL-4 engagement of IL-4R type I results in tyrosine phosphorylation of janus kinase (JAK) 1 and 3. Activated JAKs phosphorylate STAT6, which dimerizes and translocates to the nucleus of B cells to regulate target gene expression important for IgE class switch recombination and GC formation.

Our group has previously shown that GC formation was dependent on STAT6 expression in B cells during type 2 immune responses. We could further identify several STAT6-regulated genes involved in B cell proliferation and cell survival. The aim of this study was to identify new IL-4/IL-13-regulated and STAT6-dependent genes in B cells that might regulate the GC reaction. Hence, we compared the transcriptomes and proteomes of primary mouse B cells from wild-type and STAT6-deficient mice cultured for four days in the presence or absence of IL-4. We could show that CD30, CD79b, SLP-76, DEC205, IL-5Ra, STAT5 and Thy1 are induced by IL-4 in a STAT6-dependent manner. On the other hand, Syk and the Fc receptor-like protein FcRL1 were found to be down-regulated. This study provides the basis for further functional analyses of newly identified IL-4 regulated proteins in B cells that may play a role in GC formation and IgG1/IgE switching in type 2 immunity.

We also determined how STAT6 influences the migratory behavior of B cells within the spleen. For this we studied the role of STAT6 for B cell entry into and localization within the GC by two main approaches. First, we transferred WT or STAT6$^{-/-}$ (both CD45.2) naïve B cells into Sheep Red Blood Cell (SRBC)-immunized WT (CD45.1) mice on day 8 after immunization and analysed their contribution to the GC formation on day 12. Here we observed that only naïve WT B cells contributed to GC formation. Secondly, to address whether all transferred naïve B cells from WT mice migrated into GCs irrespective of their antigen-specificity we used B cells from swHEL mice. Transferred naïve B cells from swHEL mice were located only in the non-GC area of the B cell follicle. Therefore, we draw the conclusion that only antigen specific B cells can enter and migrate into GCs. The affinity maturation and differentiation into memory B cells or PCs of these “late comer GC B cells” remains to be explored.

Having established that B cell-intrinsic STAT6 is important for GC formation we further investigated how constitutively active STAT6 modulates B cell homeostasis, GC and PC formation. This was performed by characterization of CD19CreSTAT6vt mice which express a constitutively active form of STAT6 (STAT6vt) only in B cell.

1. INTRODUCTION

1.1 Immune System

The immune system protects our body from outside invaders, such as bacteria, viruses and fungi, and from toxins. The immune system is made up of different organs, cells, and molecules that all work together to prevent pathogens from causing disease in the body. The important cells of the immune system are white blood cells, also called leukocytes, which come in two basic types that combine activities to seek out and destroy disease-causing organisms or substances. The crucial task for the immune system is maintaining homeostasis as well as being tolerant to the presence of the body's own structures and proteins or so-called self-antigens. A loss of this tolerance can lead to a state in which the immune system is attacking cells and tissue of the own body, thus causing autoimmune disease (1). All these specialized immune cells depicted in the figure -1 below form parts of the immune system and provide protection against infectious diseases. This protection is called immunity and is built up by the innate and adaptive immune systems, which very much depend on each other. In addition, there are immune cells that belong to the adaptive immune system but show innate features in the way they recognize and respond to antigen (2, 3).

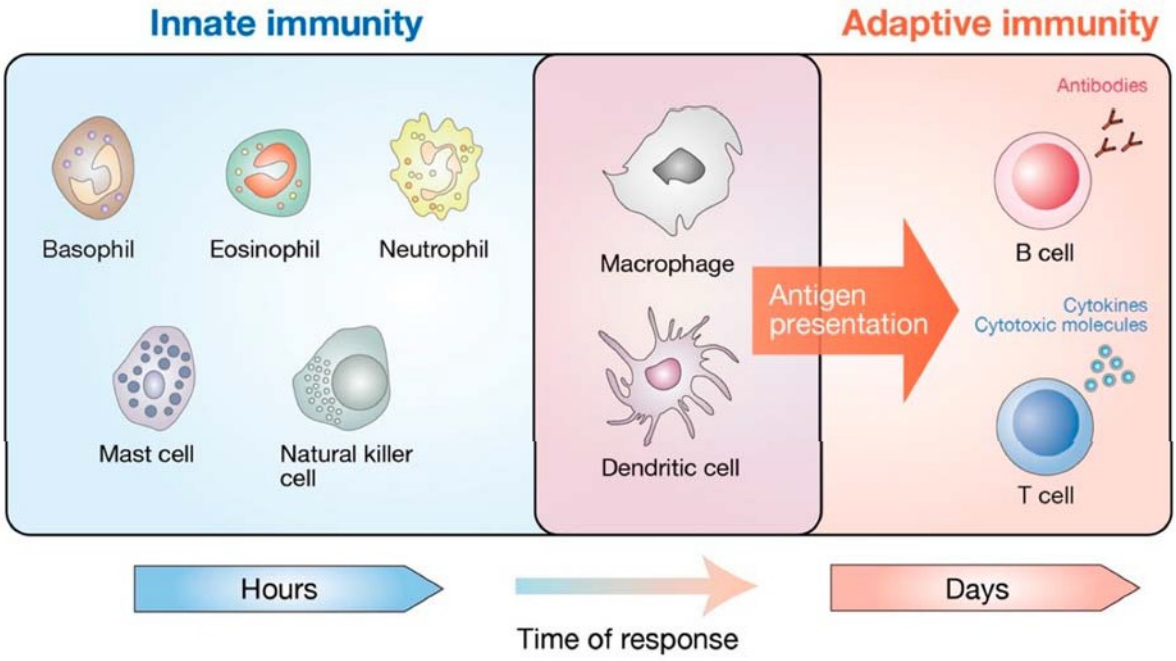


Figure.1: **Cellular components of the mammalian immune system.** The mammalian immune system consists of two distinct parts, innate and adaptive immunity. Basophils, eosinophils, neutrophils, mast cells,

natural killer cells, macrophages, and dendritic cells mediate the innate immunity. They provide the first line of defense against bacteria, viruses and cancer. The adaptive immune system includes B cell-mediated humoral immunity and T cell-mediated cellular immunity, both of which are directed towards the specific antigens. Macrophages and dendritic cells are unique subsets that have both innate and adaptive immune cell traits. Adopted from Takayoshi Yamauchi et al., 2019 [164].

1.1.1 Innate immunity

Everyone is born with innate (or natural) immunity, a type of general protection. The separation between the innate part of the immune system and the adaptive is mainly based on the kind of antigen recognition. Immune cells of the innate immune system express receptors that are encoded in the germline DNA and therefore, opposed to the adaptive immune recognition, do not require gene rearrangement. These germline-encoded receptors recognize molecular patterns such as lipopolysaccharide (LPS), lipoteichoic acid and glycans and have been conserved through evolution to protect us from pathogens with these structures. These receptors are collectively termed pattern recognition receptors (PRR) because they recognize pathogen-associated molecular patterns (PAMP) (4). A family of essential PRRs are the Toll-like receptors (TLR) some of which are membrane bound, others are intracellular, and the various family members are categorized based on what class of PAMP they bind. The TLR that was first identified as a PRR was TLR4 for its ability to bind LPS, a major component of gram-negative bacteria (5).

The cellular entity of the innate immune system is made up of monocytes, macrophages, dendritic cells (DC) and neutrophils belonging to the myeloid lineage of immune cells, as well as of natural killer (NK) cells and innate lymphoid cells (ILC). Macrophages and neutrophils are phagocytic cells which, after recognition of microbes by PRRs, can phagocytose or engulf them and subsequently destroy them in intracellular vesicles. Monocytes are abundant in the circulation and can, upon inflammation, migrate into tissues and further differentiate into macrophages. Dendritic cells are so called antigen-presenting cells (APC) as they present antigens to T cells and thereby form a very important bridge between innate and adaptive immunity. Although the DC is often appreciated for its antigen presenting capability it should be noted that also macrophages and B cells are APCs (1). Innate immunity is the initiator of inflammation, but when innate immune defenses are not sufficient to overcome the intrusion of a pathogen, more specific effector cells are needed. Cues from the innate immune system are necessary for orchestrating an appropriate adaptive immune response to a particular pathogen.

1.1.2. Adaptive immunity

Unlike the innate immune system, adaptive immune responses are carried out by white blood cells called lymphocytes. There are two classes of responses- antibody response and cell-mediated immune response; both types of lymphocytes, called B cells and T cells, carry out these responses.

In antibody responses, B cells are activated to differentiate into plasma cells (PCs) which produce specific antibodies. The antibodies circulate in the bloodstream and permeate the other body fluids, where they bind specifically to the foreign antigen that stimulated their production (Figure 1). Binding of antibody inactivates viruses and microbial toxins by blocking their ability to bind to receptors on host cells. Antibody binding also marks invading pathogens for destruction, mainly by making it easier for phagocytic cells of the innate immune system to ingest them (6).

In the second class of adaptive immune response, the cell-mediated immune response, activated T cells react directly against a foreign antigen that is presented on the surface of a host cell. The function of a T cell can be the direct killing of a virus-infected host cell that has viral antigens on its surface (Figure 1). Other types of T cells produce signal molecules that activate macrophages to destroy the invading microbes that they have phagocytosed (6).

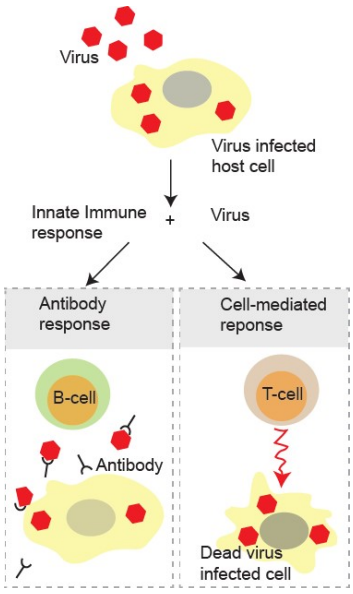


Figure.2: **Lymphocytes carry out both classes of** adaptive immune **responses.** Here, the lymphocytes are responding to a viral infection. In one class of response, B cells secrete antibodies that neutralize the virus. In the other, a cell-mediated response, T cells kill the virus-infected cells.

1.1.3 Lymphoid organs

The thymus and the bone marrow are both classified as central or primary lymphoid organs because they are home of developing immature progenitors of immune cells. The thymus is the most important organ for T cell development. When cells leave the bone marrow, they go to secondary lymphoid organs to continue their development or differentiation upon activation. Secondary lymphoid organs include lymph nodes, the GALT (gut-associated lymphoid tissue) and the spleen. The spleen contains two main regions of tissue called 'white pulp' and 'red pulp'. **Red pulp:** Is where blood is filtered through the spleen in a specialized structure of veins. **White pulp:** Mostly consists of T cells and B cells). Splenic B cells reside in an opportune location to capture and respond to blood-borne antigens, as the marginal zone is where blood is being filtered into the spleen through a sinusoid system to eventually enter the red pulp.

1.2 B cells

Early B cell development first occurs prenatally in the fetal liver, before continuing in the bone marrow. In the 1960s, Max Cooper demonstrated that antibody production was completely abrogated in irradiated chickens after surgical removal of the Bursa of Fabricius from which the notation 'B' cell was derived (7). The B cell is classically considered as a cell belonging to the adaptive part of the immune system that requires T cell help to become activated and subsequently differentiate and produce antibodies to attenuate infection. However, B cell biology is diverse, there are different B cell subsets that are located in different anatomical locations in the body, and they differ in their antigen recognition properties and activation pathways.

1.2.1 B cell development

The bone marrow is the primary location for early B cell development from stem cell to immature B cell. Development from immature to mature B cell takes place in secondary lymphoid organs. Alternative locations for early B cell development are also the fetal liver and the lamina

propria of the gut (8,9). In the B cell lineage pathway, a common lymphoid progenitor cell gives rise to a B lymphocyte progenitor that then differentiates into, a Pro-B cell, a Pre-Pro B cell and a Pre-B cell, prior to the generation of an immature B cell. These stages of B cell development are antigen-independent. Immunoglobulin gene rearrangement that occurs during these stages of development results in the expression of a mature B cell receptor (BCR) that is capable of binding to antigen. Development of mature B cells consists of a number of steps during which V(D)J recombination takes place to assemble a functional BCR (10). During V(D)J recombination, there is no control for the fact that the BCR generated from gene rearrangements should not be reactive towards self-antigens. There are however checkpoints, both in the bone marrow and the periphery to ensure that B cells are tolerant to self-antigens (10).

After V(D)J rearrangement and assembly of a functional BCR, B cells leave the bone marrow as transitional B cells, so as they are transitioning from immature to mature B cells. When B cells leave the bone marrow they acquire IgM on their surface and, upon exit, they also acquire the surface expression of IgD (11).The transitional B cells home to the spleen where they can continue their development into fully mature B cells. The developmental stages of transitional B cells into mature B cells can be distinguished using different surface markers (12, 13).

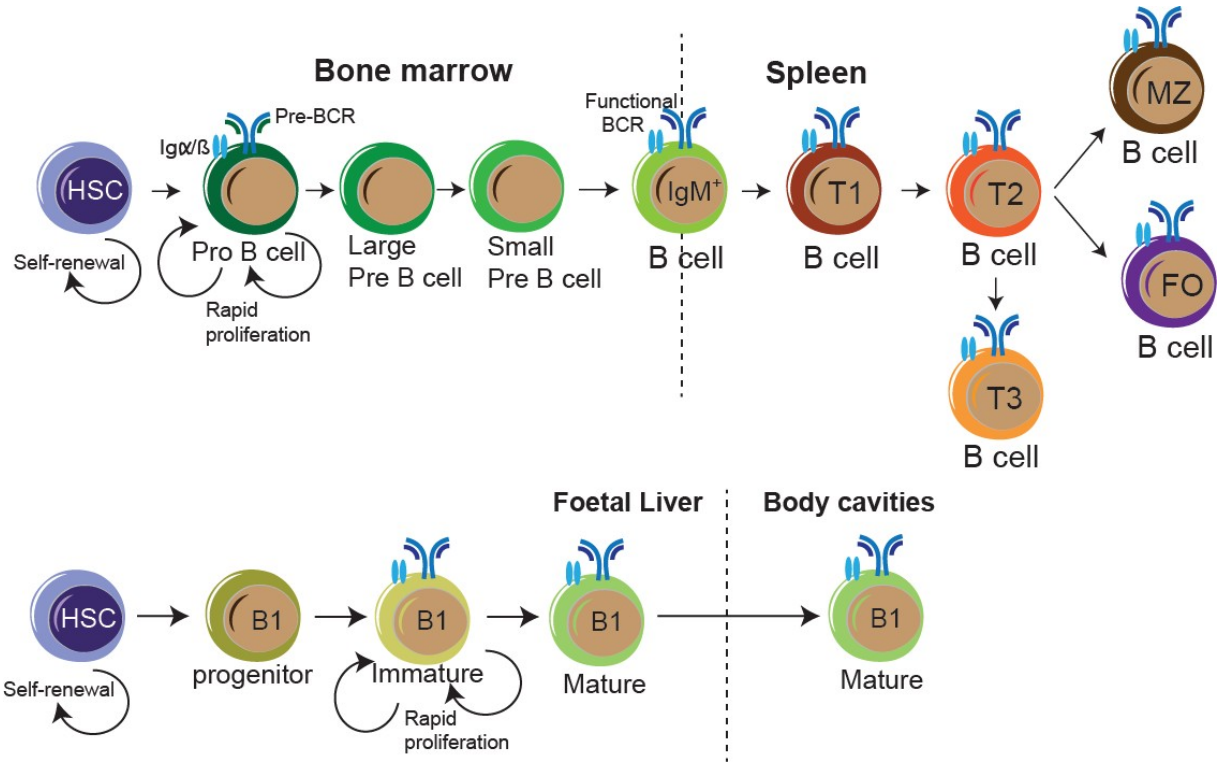


Figure.3: B-cell development and B-cell subsets. By courtesy of Ali Roghanian, University of Southampton Faculty of Medicine, UK.

1.2.2 B cell subsets

Transitional B cell stages, termed transitional type 1 (T1) and transitional type 2 (T2), are immature B cells that upon their exit from the bone marrow express IgM (the BCR). However, as T1 B cells differentiate into T2 B cells they acquire increased surface expression of IgD as well as other markers such as CD23 and CD21 (13). Evidence has also been put forth supporting the existence of a third transitional B cell population, namely transitional type 3 (T3) B cells, which can be distinguished using an additional marker; AA4 (14). Transitional B cells home to the spleen where they differentiate into mature B cells. B cells experience both antigen-dependent and -independent phases of selection, tightly regulated through signaling events.

Naïve B cells are generally divided into three subsets, B-1 B cells, FOB, and marginal zone (MZ) B cells. FOBs are the most abundant type of B cells and are, as the name implies, mainly located in B cell follicles in secondary lymphoid organs, but they also recirculate. MZBs are non-recirculating and only reside in the unique niche that is the marginal zone of the spleen. Phenotypically, MZBs are characterized by expression of high levels of CD21, CD1d and CD36 (15). CD21 on MZBs can help them to trap ICs in the marginal zone and transfer these into the follicle to deposit them on FDCs [16]. A special functional characteristic of MZBs is that they respond rapidly to blood-borne antigens and that they are able to do this without T cell help (17).

B1 cells are derived from the bone marrow but also from the fetal liver. B-1 cell development in relation to B2 development is still not fully understood and there are two different hypotheses: One claims that they stem from distinct precursors and the other one that they are derived from a common precursor (18). B-1 B cells are located in spleen and are typically subdivided into B-1a and B-1b B cells. Their common phenotype based on surface markers is CD19hiB220loCD43+CD23-IgMhiIgDlo. The B-1a subset is distinguished from the B-1b cells by expression of CD5 (8). One important characteristic of B-1 cells is their ability to produce natural antibodies, which are generated without the presence of pathogens, as they have been shown to be present in germ-free mice at steady state (19). It should however be noted that B-1 cells are

not the only source of natural antibodies, as MZBs are also capable of producing antibodies under homeostatic conditions (20).

There are also subsets of B cells with regulatory functions. B cells have a positive role in priming adaptive CD4+ T cells, but not CD8+ T cells. The magnitude of CD4+ T-cell responses is reduced upon pathogen challenge in B-cell deficient or -depleted mice. B cells are also able to dampen T-cell driven immune responses, giving rise to the concept of regulatory B cells (Breg) (7). Naïve B cells, memory B cells and MZBs can be divided by their differential expression of CD27 and IgD. CD27 is a marker for human memory B cells but is not present on immature or mature B cells, except for MZBs. IgD can then be used to distinguish between memory B cells and MZBs, where memory B cells are IgD-. There are a lot more specific surface markers for these subsets and, for instance the markers mentioned do not distinguish between transitional immature and mature B cells (21). Once activated, a B cell will differentiate into different activated B cell subsets which are the GC B cells, plasma blasts and plasma cells, and memory B cells.

1.2.3 B cell activation

Following maturation in the bone marrow and spleen, immunocompetent B cells remain in peripheral tissues until they encounter an antigen and are activated. B cell activation requires two distinct signals. The first activation signal occurs when their B cell receptor (BCR) binds to either a soluble or a membrane bound antigen. The second activation signal occurs with or without T cell help, in so called T cell-dependent (TD) responses or T cell-independent (TI) responses, respectively. The type of response depends a lot on the antigen in question, and antigens can therefore also be classified as being TD or TI. Once activated, B cells may undergo class switch recombination. In their inactivated state, B cells express IgM/IgD but once activated they may express IgA, IgE, IgG, or retain IgM expression. They achieve this by excision of the unwanted immunoglobulin isotypes. Cytokines produced by T cells and other cells are important in determining what Ig isotype the B cells express.

Upon activation, B cells proliferate and form germinal centers, where they differentiate into memory B cells or plasma cells. Following differentiation into plasma cells, additional signals initiate plasma cell antibody class-switching and regulate antibody secretion.

1.2.3.1 The germinal center

The germinal center (GC) is a specialized microstructure that forms in secondary lymphoid tissues and also supports high-affinity, long-lived humoral immunity. It consists of antigen-specific activated B cells that are undergoing clonal expansion and also manipulation of their BCRs to ultimately create B cells that are more efficient in clearing the pathogen in question (22). When a B cell meets its antigen and is initially activated, it will upregulate CCR7 (chemokine receptor 7) and, in response to CCL21 (chemokine ligand 21), migrate towards the T cell zone and in the T-B border where GCs are formed. At this point, the GC B cells upregulate an enzyme called activation-induced cytidine deaminase (AID), which together with other enzymes is responsible for somatic hypermutation (SHM) and class-switch recombination (CSR) (23). SHM is a process where random point mutations are introduced in the segments of the BCR in the variable gene-region and this results in B cells with altered affinity for the antigen. Since the mutations are random, both B cells with unchanged, higher or lower affinity for the antigen as well as self-reactive B cells can be generated. In order to select for, and further expand only the B cells with sufficiently high affinity for the antigen, there is a specialized subset of cells in the GC called FDCs (24). The presentation of antigen by FDCs to B cells in the GC reaction allows for antigen-driven selection and affinity maturation of the cells with the highest affinity for the antigen. The GC is divided into a dark zone, where proliferation and SHM takes place and following this, B cells migrate to the light zone, where the FDCs are located and antigen-driven selection takes place. B cells that don't get selected undergo apoptosis and are phagocytosed by specialized macrophages called tangible body macrophages (25). Defects in these macrophages have been linked to autoimmune disease, presumably because of the resultant accumulation of apoptotic cells (26). CSR is the other AID-induced process that takes place in a GC and as the name implies means a switch of the isotype class of the BCR which will always, for a previously antigen-inexperienced B cell, be IgM and IgD. Different antibody subclasses have different effector functions and the choice of isotype in this process depends on the cytokine milieu induced by the pathogen. It should however be mentioned, that CSR has also been shown to occur independently of AID and the GC (27).

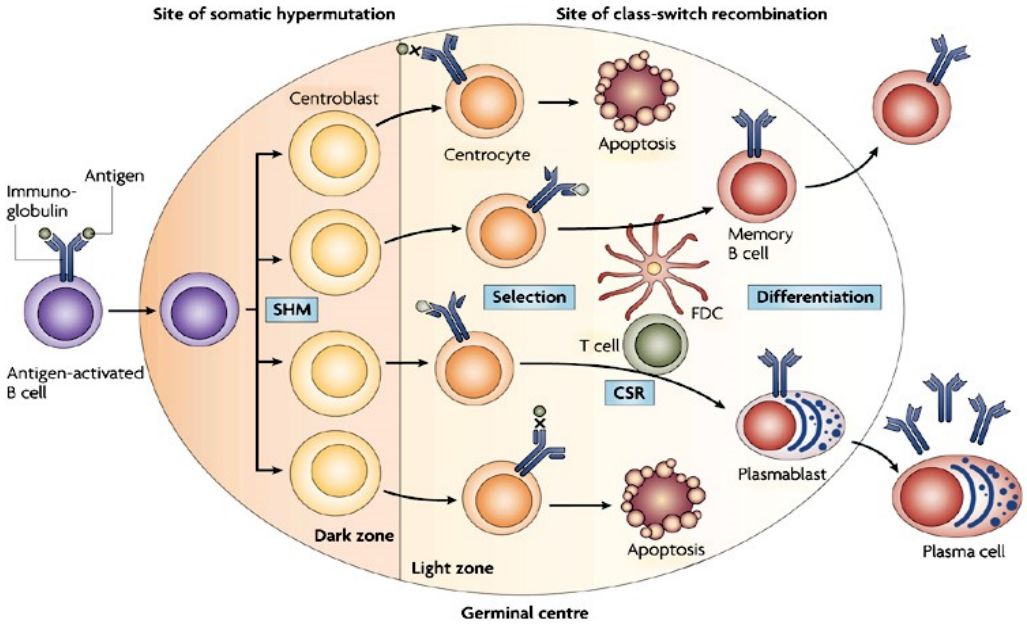


Figure 4: The germinal center microenvironment. The germinal center (GC) of lymphoid organs is the main structure where antigen-activated B cells diversify their immunoglobulin genes by somatic hypermutation (SHM) to generate high-affinity antibodies. Most of the cells also undergo class-switch recombination (CSR) to generate antibodies with specialized effector functions. Adopted from Ulf Klein et al., 2008 [165].

1.2.3.2 Plasma cells

Upon activation, B cells can differentiate to antibody-producing plasma blasts and plasma cells. Plasma cells have a slightly extended endoplasmic reticulum and a bigger cytoplasm than other B cells, as to maintain the production, storage and secretion of large quantities of antibodies (28). They are characterized by their expression of CD138 and low expression of the pan B cell marker B220, and the expression of the transcription factor Blimp-1 (29). Blimp-1 is detected in antibody-secreting cells following both T-dependent and -independent antigen challenge, in plasma cells in the bone marrow, as well as in a subset of cells in the GC displaying a pre-plasma cell phenotype. However, Blimp-1 is not detected in memory B cells (30).

Short- and long-lived population of plasma cells: B cells are activated and proliferate by the interaction between antigen-specific B and T cells. Activated B cells are differentiated into two populations: (1) differentiation into short-lived plasma cells or (2) the formation of a germinal

center and generation of long-lived plasma cells. Short-lived plasma cells are found in the secondary lymphoid tissue, while the majority of long-lived plasma cells are detected in the bone marrow. However, some long-lived plasma cells remain in secondary lymphoid tissues (30). The differentiation of activated B cells into plasma blasts at extra follicular sites is associated with the loss of activation markers on the B cell's surface such as MHCII, co-stimulatory molecules, CD19 and the BCR itself. An important factor for the maintenance of plasma blasts and their subsequent differentiation into *bona fide* plasma cells in extra follicular foci has been shown to be a specific DC population located in these sites that expresses high levels of CD11c (31).

1.2.3.3 Memory B cells

Another outcome of B cell activation, following both a TI and TD response, and in both the follicular GC and extra follicular pathway, is the generation of memory B cells. As the name implies, upon re-encounter with a pathogen the existence of memory B cells is essential for the efficient development of neutralizing antibody responses (32). The classical view of the phenotype of a memory B cell is that its BCR is isotype-switched and of high affinity for the antigen, as it has been generated through CSR and SHM processes in a GC reaction. However, new evidence has emerged showing that there are also GC-independent memory B cells (33, 34). The protein SAP (signaling lymphocyte activation molecule associated protein) has been shown to be important for the duration of the T-B cell contact at this stage and may therefore be also involved in the fate decision (35). Further, CD40 signaling and regulation of Bcl6 by the cytokine IL-21 have been implicated in affecting the choice of pathway (33, 36). Recently, a study showed that memory B cells and long-lived plasma cells are generated at different time points during the course of a GC reaction and even that memory B cells with different effector functions are generated at different time points (37). Affinity for the antigen is also important for the choice between differentiation into a memory B cell or a long-lived plasma cell, where high-affinity B cell clones have a propensity for differentiating into plasma cells rather than memory B cells (38). In addition to memory B cells developed through varying degrees of T-B cell interactions, memory B cells can also be generated from TI responses. Notably, B1 cells have been shown to generate memory B cells in a TI manner, although the recall response in this case, as compared to TD memory, was more qualitative than quantitative (39). In summary, the quality and features of a memory response will vary depending on involvement of a previous GC reaction, the level of T cell help if any, the type of B cell activated and the type of antigen.

1.3 The JAK/STAT pathway

Upon cytokine stimulation, receptors undergo conformational changes that bring JAKs into proximity with each other, enabling their activation through tyrosine transphosphorylation (40). To date, four mammalian members of the JAK family have been cloned (JAK1, JAK2, JAK3 and TYK2) and as a draft sequence of the human genome has now been completed the number is not expected to increase (41). JAKs are relatively large protein kinases (110-140 kDa) that are ubiquitously expressed, with the exception of JAK3, whose expression is restricted mainly to hematopoietic cells (42). Studies with gene-targeted mice and in humans with JAK3 deficiency have attested the non-redundant in-vivo function of JAK family members (43). The results from JAK knockout experiments are also in line with the reported functions of different cytokine receptor chains, thus confirming the essential role of JAKs in cytokine signaling.

Based on sequence conversation, the structure of JAK kinases can be divided into seven JAK homology (JH) domains. The most characteristic feature of JAK kinases is their unique tandem-kinase structure composed of a tyrosine-kinase domain (JH1) and a pseudokinase domain (JH2, alternatively termed also kinase-like domain) (44, 45). The JH1 domain possesses all the typical features of tyrosine kinase domains and it is catalytically active (46, 47, 48). The pseudokinase region has sequence similarity with kinase domains, but many residues that are required for phosphotransferase activity are altered, and consequently the JH2 domain is catalytically inactive (49). The pseudokinase domain has been implicated in the autoregulation of JAK kinase activity (47, 48, 50). In addition to ligand-induced autophosphorylation, JAKs also phosphorylate intracellular tyrosine residues on cytokine receptors, thus creating docking sites for proteins that contain an SH2 domain, such as STAT transcription factors. Attached STATs also become tyrosine phosphorylated by JAK kinases, which results in the homodimerization of STATs. Once activated, dimerized and tyrosine phosphorylated, STATs rapidly translocate to the nucleus where they bind to the promoters of cytokine responsive genes (51). The DNA-bound STATs have an intrinsic ability to recruit nuclear co-activators and subsequently induce the transcription of target genes.

1.3.1 STATs

The transcription factors of the STAT family were initially recognized as ligand-induced transcription factors in IFN-treated cells. In human, STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) are encoded by seven genes mapped on chromosomes 2, 12 and 17 (52). Through structural function analysis it has become evident that all seven STAT family members share six conserved domains: N-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, Src homology (SH) 2-domain and carboxy (C) terminal transactivation domain (TAD).

In the promoter regions of several cytokine-inducible genes, two STAT-binding sites are located about 20 base pairs apart from each other, and these can be occupied by tetramerized STAT dimers. The 130 conserved N-terminal residues have been implicated in the tetramerization of STATs, and consequently they further stabilize the STAT-DNA interactions (45, 53). The N-terminal part has also a role in receptor recognition and in the regulation of the nuclear transport and dephosphorylation of STATs (54). The coiled-coil domain has also been demonstrated to take part in the nuclear export of STATs (45).

The STAT DNA binding domain positioned C-terminal to the coiled-coil domain (residues ~320 to 490) contains several β-sheets that are folded similarly to the DNA binding domains of nuclear factor (NF) κB and p53 (53). The adjacent linker domain (amino acids ~490 to 580) connects the DNA binding domain with the SH2 domain and has also been implicated in nuclear export (45). The SH2 domain recruits STATs to tyrosine-phosphorylated receptor units and is also involved both in the association of STAT with the activating JAK kinase and in STAT dimerization (55, 56). The SH2 domain (amino acids ~580 to 680), the most highly conserved region of STATs, is a pocket-like structure of antiparallel β-sheets flanked by two α-helices. A conserved arginine residue that mediates interactions with phosphate is positioned at the base of this pocket (45).

1.3.2 STAT-DNA binding

As discussed above, in most cases, a cytokine stimulus results in the activation and homodimerization of STAT proteins. In some occasion, heteromers between STATs 1 and 3, and 5A and 5B are also known to be formed (57). Activated STAT dimers recognize a palindromic motif defined as (Interferon gamma) activated site (GAS) with a consensus

sequence of 5'- TT(N4-6)AA-3'. The most preferable spacing between AA and TT residues varies between the different STAT family members. STAT1 binds a DNA element with five spacing nucleotides (n=5), whereas STAT3 and STAT6 favor elements where n=4 and n=6, respectively (58, 45). Though the structure of full length STAT remains yet to be elucidated, studies with DNA-bound STAT1 and 3 molecules have suggested a nutcracker-like structure to be established between dimerized STATs and DNA (53, 42).

As a notable exception, STAT2 does not bind DNA as a homodimer, but functions as a component of the multimeric IFNα-induced IFN-stimulated gene factor 3 (ISGF3) complex (59). The ISGF3 complex, consisting of STAT1, STAT2 and IRF9 transcription factor, recognizes a unique IFNα stimulated response element (ISRE, consensus sequence 5'-AGTTN3TTTCC-3') but fails in binding to GAS elements (57).

1.4 IL-4/STAT6-mediated gene activation

1.4.1 Interleukin-4

IL-4 is a pleiotropic cytokine predominantly produced by mast cells, T helper (Th2) cells and basophils. IL-4 is implicated in the regulation of growth and function of various cell types, including B and T lymphocytes, eosinophils, keratinocytes, epithelial cells, hepatocytes and myeloid cells (60). The IL-4 gene, which encodes a cDNA for a glycoprotein of 129 amino acids, is mapped in the human cytokine cluster found on chromosome 5q23-31 (61). In the hematopoietic cell compartment, IL-4 functions as a key regulator of humoral immune responses executed by Th2 and B lymphocytes.

In contrast to its original and well-established role as a B cell growth factor, in pre-B leukemia cells IL-4 has been shown to promote apoptosis (62, 63, 64). The B cell differentiation stage thus serves as an important determinant for the biological outcome of IL-4 treatment. Notably, an IL-4 stimulus alone is not sufficient to drive the deletion switch-recombination of immunoglobulin, but a second signal must also be provided to B cells by surface antigen CD40 engagement (65). IL-4 also induces the expression of other immunomodulatory genes in B cells, such as major histocompatibility complex class II (MHC II) and the low affinity receptor for IgE (FcεRII or CD23) (66).

The fate of antigen-activated naïve T cells (TH0) is dictated by the surrounding cytokine milieu. For T cells, IL-4 is generally considered as a hallmark cytokine for humoral immunity due to its potential of skewing T helper cells into TH2 direction. IL-4 is capable of upregulating its own production and it acts as an autocrine growth factor for TH2 cells. Simultaneously, IL-4 suppresses the IFNγ-driven TH1 polarization and thereby the vents of cell-mediated immunity (67). IL-4 also directly inhibits the monocytic expression of proinflammatory cytokines like IL-1, IL-6 and tumor necrosis factor (TNF) (68).

IL-4 deficient mice have normal B and T cell development but greatly diminished levels of IgG1. Moreover, they completely lack IgE (69). Some of the biological functions of IL-4 can be substituted by the functionally related cytokine IL-13. The IL-13 gene is located within 50 kb of the IL-4 gene and similarities in the intron-exon structure suggest evolution from a common ancestral gene by gene duplication (68, 70, 71). Although IL-4 and IL-13 share only 25% homology in primary structure, their biological properties are largely overlapping, mainly due to a shared receptor subunit. However, differences both in the kinetic of cytokine secretion as well as in the regulation of immune response against parasitic infection have been reported, and the biological effects of IL-13 are generally more restricted than those of IL-4 (72). Emerging evidence has also suggested that IL-13 has a specific, IL-4-independent role in the pathogenesis of bronchial asthma (73, 74).

1.4.2 The IL-4 receptor complex

The receptors for IL-4 are ubiquitously expressed in a wide variety of cell types (75). The IL-4 receptor complex is predominantly composed of the ligand-specific IL-4 receptor α-chain (IL-4Rα) and the common γ-chain (γc). Alternatively, IL-4Rα forms heterodimers with the IL-13 receptor alpha chains (IL-13Rα and IL-13Rα'), and in some circumstances two copies of IL-4Rα can also homodimerize (75, 76). Because of the usage of a common subunit shared by the receptors for interleukins 2, 4, 7, 9, 15 and 21, the IL-4 receptor is logically categorized into the γc-family of cytokine receptors. The redundant functions of IL-4 and IL-13 cytokines are largely explained by the existence of IL-4Rα-IL-13Rα or IL-4Rα-IL-13Rα' receptor heterodimers, which can activate cellular signaling in response to both cytokines (77, 78).

The IL-4Rα gene in human encodes a 140-kDa glycoprotein with typical features (e.g. a WSXWS motif in the extracellular portion and the Box1 and Box2 motifs in the intracellular domain) of a

cytokine receptor (40, 79). The cytoplasmic domain of IL-4Rα contains five highly conserved tyrosine residues with an important function in IL-4-induced cellular signaling. The most membrane-proximal Tyr497 mediates proliferative signals while Tyr575, Tyr603 and Tyr631 are important for STAT6 activation, and the C-terminal Tyr713 serves as a docking site for phosphatases (77). Interestingly, mutations in IL-4Rα have been reported to have clinical significance. Ile50Val substitution in the IL-4Rα extracellular motif has been linked to increased IgE production and atopic diseases (80), and a polymorphism Gln579Arg in the intracellular core STAT6-binding region has been found in patients with hyper-IgE syndrome and atopic dermatitis (81).

Similar to IL-4Rα, the extracellular part of γc also shows typical characteristics of a cytokine receptor. The gene encoding γc, a 64-kDa glycoprotein, is widely expressed in hematopoietic cells but generally absent in other cell types. The critical role of γc in the development of lymphocytes is well illustrated by individuals suffering from severe combined immunodeficiency (SCID) on the basis of functionally mutated γc (82). Two different human IL-13 receptor α chain variants (IL-13Rα and IL-13Rα' or alternatively termed IL-13Rα1 and IL-13Rα2) have been cloned (83, 84). Both components are 55-70kDa in size and they bind IL-13 with different affinities. IL-13Rα initially binds IL-13 with the subsequent recruitment of IL-4Rα to efficiently transduce a signal. IL- 13Rα' can bind IL-13 in the absence of IL-4Rα, but its role in IL-13 signaling is still unclear. Importantly, IL-13Rα' is reported to serve as a component of the functional IL-4 receptor complex in γc-deficient, non-hematopoietic cells (85).

1.4.3 Immediate signaling events upon IL-4 stimulus

An IL-4 stimulus results in the dimerization of the IL-4 receptor chain. Characteristical for cytokine receptors, none of the components of the IL-4R complex has intrinsic kinase activity; rather the activation of associated tyrosine kinases is required for the initiation of signaling events. All the members of JAK kinase family have been shown to be activated upon IL-4 stimulus. JAK1 and (in certain cell lines) JAK2 are shown to be associated with the IL-4Rα chain, while JAK3 is exclusively bound to γc (86, 75). When IL-4 signaling occurs through an IL-4Rα-IL-13Rα complex, TYK2 kinase as another IL-13Rα associated JAK-family member can also be activated (87).

Bound to dimerized receptor chains, JAKs get rapidly activated and transphosphorylated followed by the phosphorylation of critical tyrosine residues in the cytoplasmic domains of the IL-4 receptor complex.

A motif (amino acids 437 and 557) present in the membrane-proximal cytoplasmic region of IL-4Rα mediates the recruitment and activation of IRS proteins and is therefore designated 'insulin IL-4 receptor (I4R) motif'. Four IRS proteins (IRS-1-4) with at least partly redundant functions have been cloned (88). IRS proteins are large adapter proteins (approximately 170 kDa) that become tyrosine phosphorylated by JAKs on multiple (approximately 20) tyrosine residues upon cytokine treatment (89). The phosphotyrosines on IRS proteins serve as docking sites for SH2 domain containing signaling molecules, including the regulatory subunit of phosphoinositide-3-kinase (PI-3-K) and the Ras/MAPK pathway adaptor molecule Grb-2 (89). IRS downstream signaling via activated PI-3-K and its substrate Akt/protein kinase B is implicated in the regulation of IL-4- mediated cell growth and the prevention of apoptosis (90, 91).

1.5 Structure of STAT6

STAT6 (previously also termed as IL-4 STAT, STF-IL-4 and IL-4 NAF) was originally identified as a GAS-binding protein in extracts derived from IL-4-treated cell lines (92, 93, 94). In addition to IL-4, the functionally related cytokine IL-13 is able to trigger STAT6 activation (95). More unconventional stimuli that activate STAT6 include angiotensin II, platelet-derived growth factor (PDGF), leptin and surface Ig or CD40 crosslinking (77). The biological significance of STAT6 activation in these alternative pathways remains unclear.

The STAT6 gene is mapped to the human chromosome 12 (12q13-14.1) and to murine chromosome 10. It encodes a single 4 kb transcript for a 94 kDa protein (96, 97). STAT6 shares a 34% structure homology with STAT5, but is less similar to the other STAT family members (17-20% homology). STAT6 harbors the typical functional domains of all STAT family members. The functionally indispensable tyrosine phosphorylation upon IL-4/IL-13 stimulus occurs at Tyr641 positioned adjacent to the SH2 domain (58). The substitution of two residues at positions 547 (Val) and 548 (Thr) within the SH2 domain by alanine has reportedly resulted in an IL-4-stimulus-independent, constitutively active STAT6 mutant. Thus, small changes in the STAT6 gene can cause hyperactivation of the protein and thereby the constitutive expression of STAT6-dependent genes (98). The most unique feature of the STAT6

structure is formed by 170 C-terminal amino acids that serve as an autonomous transactivation domain TAD (58). As the TADs of other family members, STAT6TAD is also rich in proline residues and contains eight serine residues that are positionally conserved between mouse and human species (99). However, compared to the TADs of STATs 1, 3, 4 and 5, STAT6TAD is considerably larger and lacks the functionally important consensus phosphorylation site for MAPK kinases (57, 100). Two distinct portions of the STAT6 C-terminus (amino acids 661-715 and 753-810) are able to activate IL-4 responsive reporter constructs when fused as a duplicate with a C-terminally truncated STAT6 mutant (101). Another proof of the IL-4- independency of STAT6TAD in the initiation of transcription came from experiments with conditionally active STAT6-estrogen receptor (ER) chimera. Estrogen treatment results in the dimerization and nuclear translocation of STAT6-ERs, which consequently results in the activation of transcription without IL-4 treatment or Tyr641 phosphorylation (102).

In addition to the full-length STAT6 proteins, naturally existing isoforms on the basis of alternate splicing of mRNA have also been described. STAT6b has a deletion at the N-terminus while STAT6c lacks a portion of SH2 domain. Both isoforms can reduce IL-4 response when overexpressed in cells (103).

1.5.1 Biological impact of STAT6

Several lines of studies in-vitro have demonstrated the essential role of STAT6 as mediator of IL-4/IL-13-induced gene responses. The analysis of mice with targeted STAT6 gene disruption has further illuminated the profound impact of STAT6 on the expression of IL-4 inducible genes *in vivo* (104, 105, 106). Lymphocytes isolated from $STAT6^{-/-}$ mice fail to express MHC II, CD23 and IL-4Rα genes upon IL-4 stimulation. STAT6-deficient B cells do not produce detectable levels of IgE, and the IgG1 production is also greatly diminished. Th cells (T helper cells) isolated from STAT6 deficient animals do not differentiate into the TH2 phenotype under a proper skewing cytokine environment. Furthermore, in conflict with *in vitro*- findings highlighting the role of IRS proteins in IL-4-induced cell proliferation, studies with gene-targeted mice uncovered the function of STAT6 also in the regulation of mitogenesis (90, 104). Cell cycle analysis showed that STAT6-deficient lymphocytes are defective in the progression from the G1 to the S phase

of the cell cycle due to significantly increased expression levels of the cyclin-dependent kinase (CDK) inhibitor p27Kip 1 protein (107).

STAT6-deficient animal models have also enabled a more clinically-oriented evaluation of the function of STAT6 in pathogenesis in experimental models of human disease. $STAT6^{-/-}$ mice fail to expel helminthic parasites such as *Nippostrongylus brasiliensis* and have an exaggerated severity of Th1-type disease due to the absence of Th2 type responses (108, 109). The predominance of cell-mediated Th1-type response also results in increased tumor immunity (110, 111, 112). Much effort has been aimed at assessing how STAT6 is implicated in the pathogenesis of atopic diseases, asthma, and allergy. In the lungs, STAT6-deficiency downregulates experimental allergen-induced inflammation, bronchial hyper-responsiveness and mucus overproduction (113, 114). The involvement of STAT6 in the pathogenesis of asthma is also suggested by more indirect evidence. Patients with elevated STAT6 expression levels in airway have a more severe disease outcome (115).

In genetic linkage studies with atopic diseases the exons, flanking introns and the promoter region of the STAT6 gene have been screened for single nucleotide polymorphisms (SNPs). Association between a GT repeat in allele A4 and enhanced eosinophilic inflammation, and a link of a SNP in intron 18 and an increase in total IgE levels were found (116). However, neither the SNP nor the GT repeat showed linkage/association to asthma as a disease. In contrast, another study has shown that polymorphism in the 3' untranslated region (UTR) of the STAT6 gene is associated with the susceptibility to and the severity of nut allergy (117).

2. AIMS OF THE STUDY

Infection with helminths induces a strong type 2 immune response resulting in the secretion of the cytokines IL-4 and IL-13 by CD4+ T cells and several innate cell types. These cytokines promote immunoglobulin class switching in B cells to IgG1 and IgE which contributes to the expulsion of intestinal parasites. IL-4 engagement of IL-4R type I results in tyrosine phosphorylation of Janus kinases (JAK) 1 and 3. Activated JAK kinases phosphorylate signal transducer and activator of transcription 6 (STAT6), which dimerizes and translocates to the nucleus to regulate target gene expression with importance for germinal center (GC) formation and class-switch recombination to IgG1 and IgE.

The first aim of this study was to identify new IL-4/IL-13-regulated and STAT6-dependent genes in B cells that might regulate the GC reaction.

Hence, we compared the transcriptomes and proteomes of primary mouse B cells from wild-type and STAT6-deficient mice cultured for four days in the presence or absence of IL-4.

We further determined the requirement of STAT6 for recruitment of B cells into existing GCs using adoptive transfer experiments and SRBC immunization. Finally, we created a new mouse line to investigate how a constitutively active version of STAT6 affects the development of B cells and expression of IL-4-regulated genes.

3 MATERIAL

3.1 Equipment and consumable materials

Table 1: Equipment

Equipment / Type	Manufacturer
Centrifuge 1-15PK	Sigma, Osterode am Harz, Germany
Centrifuge 3-30K	Sigma, Osterode am Harz, Germany
Centrifuge 4-16K	Sigma, Osterode am Harz, Germany
Cryostat	Lecia Biosystem, Wetzlar, Germany
Flow cytometer / Canto II	BD Bioscience, San Jose, CA, USA
Gel chamber	Thermo Fisher Scientific, Dramstadt, Germany
Galaxy 170R CO2 Incubator	Eppendorf, Hamburg, Germany
ChemiDoc MP imager	Biorad, Hercules, CA
AxioVert A1Microscope	Carl Zeiss, Oberkochen, Germany
Cellometer Auto T4 cell counter	Nexcelom Bioscience, Lawrence, MA
pH-meter Five easy	Mettler-Toledo, Columbus, OH
CFX Connect Real-time PCR Detection system	Biorad, Hercules, CA
PIPETBOY	Integra bioscience, Wallisellen, Switzerland
Pipettes and multichannel pipettes	Eppendorf, Hamburg, Germany
MS3 digital vortexer	IKA, Staufen, Germany
MSC-Advantage TM biological safety cabinet	Thermo Fisher Scientific, Waltham, MA
Western blot – Trans blot Turbo Transfer system	Biorad, Hercules, CA

Table 2: Computer software

Software	Manufacturer
FACS Diva	BD Bioscience, San Jose, CA, USA
FlowJo v.10	Treestar, Ashland, OR, USA
SigmaPlot 12.3	Systat software Inc., San Jose, CA, USA
Adobe Illustrator CS5	Adobe System, San Jose, CA
Office 2016	Microsoft Albuquerque, NM

Table 3: Consumable Materials

Product	Manufacturer
Cell culture plates (flat bottom; 24-, 96-well)	Sarstedt, Nuembrecht, Germany
Coverslips (18x18 mm)	Roth, Karlsruhe, Germany
Cell culture flasks Nunc (75 cm2, 175 cm2)	Thermo Fisher Scientific, Waltham, MA
Plastic tubes (15 mL and 50 mL)	BDFalcons, Franklin Lake, NJ, USA
Cell Strainer (40 µm and 70 µm)	VWR, Darmstadt, Deutschalnd
Syringes (1 mL, 5 mL, 10 mL and 20 mL)	Braun, Melsungen, Germany
NuPAGE 4–12% Bis-Tris Protein Gels	Thermo Fisher Scientific, Dramstadt, Germany
Omnican syringes (1 mL)	Braun, Melsungen, Germany
Petri dishes	Sarstedt, Nuembrecht, Germany
Pipette tips (1-200 µl and 100-1000 µl)	Sarstedt, Nuembrecht, Germany
Plastic pipettes (5 mL, 10 mL and 25 mL)	Sarstedt, Nuembrecht, Germany
Sterile pipette tips (0.1-10 µL, 1-20 µL, 1-200 µL and 100-1000 µL)	Sarstedt, Nuembrecht, Germany
Tubes (PCR) 0.2 mL	Biorad, Hercules, CA
Tubes (1.5 mL and 2 mL)	Brand, Wertheim, Germany
Tubes (5 mL, flow cytometry)	Sarstedt, Nuembrecht, Germany

3.2 Regents and chemicals

Table 4: Reagents

Product	Manufacturer
Acetic acid 100%	Roth, Karlsruhe, Germany
Acetone >99.8%p.a.	Roth, Karlsruhe, Germany
Agarose	Roth, Karlsruhe, Germany
Albumin Fraction V > 98% biotin free (BSA)	Roth, Karlsruhe, Germany
Ammonium chloride	Roth, Karlsruhe, Germany
β -Mercaptoethanol	Thermo Fisher Scientific, Dramstadt, Germany
Calcium phosphate	Roth, Karlsruhe, Germany
Chloroquine diphosphate salt	Sigma-Aldrich, St. Louis, MO, USA
Coomassie 2G	Sigma-Aldrich, St. Louis, MO, USA
4', 6'- Diamindino-2-phenylindol (DAPI)	Sigma-Aldrich, St. Louis, MO, USA
Deoxycholic acid	Sigma-Aldrich, St. Louis, MO, USA
Ethanol > 99.8%	Roth, Karlsruhe, Germany
Ethylenediaminetetraacwtic acid (EDTA)	AppliChem, Dramstadt, Germany
Fetal Calf Serum (FCS)	Thermo Fisher Scientific, Dramstadt, Germany
Isopropanol (> 99.8%)	Roth, Karlsruhe, Germany
L-Glutamine	Thermo Fisher Scientific, Dramstadt,

	Germany
Lipopolysaccharide (LPS)	Sigma-Aldrich, St. Louis, MO, USA
Magnesium chloride	Sigma-Aldrich, St. Louis, MO, USA
Methanol	Roth, Karlsruhe, Germany
Nonidet –P- 40	Sigma-Aldrich, St. Louis, MO, USA
Penicillin	Biochrom AG, Berlin, Germany
Phosphate buffered saline (PBS)	Biochrom AG, Berlin, Germany
Polybrene (Hexa-dimethrin bromide)	Sigma-Aldrich, St. Louis, MO, USA
Postassium hydrogen carbonate	Roth, Karlsruhe, Germany
Protease Cocktail Tablets	Roche Diagnostics, Rotkreuz, Switzerland
Phosphatase Inhibitor Cocktail	Roche Diagnostics, Rotkreuz, Switzerland
Sodium chloride	Roth, Karlsruhe, Germany
Sodium-ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe, Germany
Sodium hydrogen phosphate	Roth, Karlsruhe, Germany
Streptomycin	Biochrom AG, Berlin, Germany
Tissue freezing medium	Jung, Nussland, Germany
Tris chloride	Roth, Karlsruhe, Germany
Trypan blue solution	Sigma-Aldrich, St. Louis, MO, USA
Trypsin/EDTA 100x	Invitrogen, Carlsbad, CA, USA
Tween-20	Sigma-Aldrich, St. Louis, MO, USA

Table 5: Commercial kits

Product	**Manufacturer**
EasySep™ Mouse B Cell Isolation Kit (negative selection)	Stem cell technologies, Köln, Germany
MojoSort™ Mouse Pan B Cell Isolation Kit	Biolegend, Koblenz, Germany

Table 6: Cytokines

Product	**Manufacturer**
IL-4	R&D Systems, Minneapolis, MN, USA

Table 7: Enzymes

Product	**Manufacturer**
DNase I	Sigma-Aldrich, St. Louis, MO, USA
Taq-polymerase	Genecraft, Cologne, Germany

3.3 Buffers and cell culture media

Table 8: Media

Cell culture media	Composition
B cell culture medium	RPMI 1640 20%FCS (v/v) 25mM Hepes 10mM β-Mercaptoethanol 2mM L-Glutamine 100 µg/mL Penicillin/Streptomycine

Table 9: Buffer

Solution	Composition
ACK buffer	8.29 g NH_4Cl 1g $KHCO_3$ 37.2 mg Na_2EDTA H_2O ad 1 L pH 7.2-7.4
Acid buffer	0.085 M NaCl, 0.005 M KCl, 0.01 M EDTA, 0.05 M NaAcetate pH 4
DNase buffer	0.15 M NaCl 4.2 mM MgCl in H_2O (pH 5)
Fluorescence activated cell sorter (FACS) blood buffer	2% FCS 0.1% NaN_3 2000 U/L Heparin
FACS buffer	2% FCS 0.1% NaN_3
PBS	9.55 g/L PBS
RPMI medium	RPMI 1640 10% FCS (v/v) 5x10-5 mM β-Mercaptoethanol 2 mM L-Glutamine 25 mM HEPES 100 µg/mL Penicillin/Streptomycin

RIPA lysis buffer	1% Nonidet –P-40 50 mM Tris (pH 7.4) 0.15 m NaCl 1 mM EDTA pH 8 0.25% deoxycholic acid
Stripping buffer	62.5 mM Tris-Hcl pH6.8 2% SDS 100 mM β-Mercaptoethanol
6x sample buffer	20% SDS 5% β-Mercaptoethanol 30% glycerol
5x SDS running buffer	30 g Tris, 150 g Glycine with ddWater to 1 liter.
50 x TAE	242 g Tris 57.1 mL Acetic acid (100% (v/v)) 100 mL 0.5 m EDTA (pH 8.0), H2O ad 1 L
0.1 M Tris buffer (pH7.5)	12.1 g/L Tris pH 7.5 with fuming HCl
Western blot blocking buffer	1xTBS + 2%-5% BSA + 0.1% Tween

All reagents, buffers and media were prepared with double distilled water.

3.4 Antibodies

Table 10: Anti-mouse antibodies

Anti-mouse	Species/Isotype	Clone	Conjugation	Manufacturer
CD5	Rat IgG2a	53-7.3	PerCP-Cy5.5	eBioscience, San Diego, CA, USA
CD11b	Rat IgG2b	M1/70	FITC	Biolegend, Koblenz, Germany
CD11b	Rat IgG2b	M1/70	APCe780	eBioscience, San Diego, CA, USA
CD21/CD35	Rat IgG2a	7G6	APC	BD Bioscience, San Jose, CA, USA
CD23	Rat IgG2a	B3B4	PE-Cy7	eBioscience, San Diego, CA, USA
CD30	Armenian hamster IgG	mCD30.1	PE	eBioscience, San Diego, CA, USA
CD38	Rat IgG2a	90	PE-Cy7	Biolegend, Koblenz, Germany
CD45.1	Rat IgG2a	A20	PE	eBioscience, San Diego, CA, USA

CD45.2	Rat IgG2a	104	PerCP-Cy5.5	eBioscience, San Diego, CA, USA
CD45R	Rat IgG2a	RA3-6B2	PBe450, APCe660	eBioscience, San Diego, CA, USA
CD45R	Rat IgG2a	RA3-6B2	PerCP-Cy5.5	Biolegend, Koblenz, Germany
CD79b	Armenian hamster IgG	HM79-12	PE	eBioscience, San Diego, CA, USA
CD90.2	Rat IgG2b	30H12	Allophycocyanin	eBioscience, San Diego, CA, USA
c-Kit (CD117)	Rat IgG2b	2B8	PE	eBioscience, San Diego, CA, USA
CD138	Rat IgG2a	281-2	APC	BD Bioscience, San Jose, CA, USA
IL-5Ra (CD125)	Rabbit IgG	T21	PE	eBioscience, San Diego, CA, USA
DEC205	Rat IgG2a	NLDC-145	PE	eBioscience, San Diego, CA, USA
FcRL1	Rat IgG1	745916	Allophycocyanin	R&D system, Minneapolis, MN, USA
GL-7	Rat IgM	GL-7	FITC, APCe660	eBioscience, San Diego, CA, USA
TACI (CD267)	Rat IgG2a	ebio8F10-3	PE	eBioscience, San Diego, CA, USA
IgG1	IgG1	A85-1	APC	BD Bioscience, San Jose, CA, USA
IgG1	IgG1	RMG1-1	PE	Biolegend, Koblenz, Germany
IgG2a	IgG2a	8.3	Biotinylated	BD Bioscience, San Jose, CA, USA
IgE	Rat IgG1	23G3	PE	eBioscience, San Diego, CA, USA
IgE	Rat IgG1	R35-72	FITC	BD Bioscience, San Jose, CA, USA
IgM	Rat IgG2a	RMM-1	PE	Biolegend, Koblenz, Germany
IgD	Rat IgG2a	11-26c	Biotinylated	eBioscience,San Diego, CA, USA

Table 11: Isotypes

Species/Isotype	Conjugation	Manufacturer
Rat IgG2a	PE	eBioscience, San Diego, CA, USA
Rat IgG2a	Biotinlyated	eBioscience, San Diego, CA, USA

Table 12: Streptavidin for flow cytometry

Conjugation	Manufacturer
Pacific blue	Life Technologies by Thermo Fisher Scientific

3.5 Western blot antibodies

Table 13: Primary antibodies

Antibodies	Species	Source	Dilution	Manufacturer
SLP-76	Human/Mouse	Rabbit	1:1000	Cell Signaling technology, Danvers, MA, USA
Syk	Human/Mouse	Rabbit	1:1000	Cell Signaling technology, Danvers, MA, USA
STAT5a/b	Human/Mouse	Polyclonal Rabbit IgG	1 µg/mL	R&D system, Minneapolis, MN, USA
β - actin	Human/Mouse	Rabbit IgG	1:1000	Cell Signaling technology, Danvers, MA, USA
STAT6 Cat # 35397	Human/Mouse	Rabbit IgG	1:1000	Cell Signaling technology, Danvers, MA, USA

Phospho-STAT6 Cat#9361	Human/Mouse	Rabbit	1:1000	Cell Signaling technology, Danvers, MA, USA

Table 14: Secondary antibodies

Antibodies	Conjugation	Dilution	Manufacturer
HRP	Donkey Anti-Rabbit IgG	1:5000	Jackson ImmunoResearch, Cambridgeshire, UK

3.6 Biological material

Table 15: Mouse strains

Mice	Description	Source
BALB/c	BALB/c mice with the MHC haplotype H-2d were used as wild type control mice.	The Jackson Laboratory, Bar Habor, ME, USA
C57BL/6	C57BL/6 mice with the MHC haplotype H-2b were used as wild type control mice.	The Jackson Laboratory, Bar Habor, ME, USA
STAT6-/-	The Stat6-deficient mice were generated by replacing the two exons of the Stat6 gene with the neomycine resistance gene.	The Jackson Laboratory, Bar Habor, ME, USA
CD19Cre	CD19cre is a transgenic mice in which express cre under the transcriptional control of the B lineage-restricted CD19 gene	Franz-Penzoldt-Zentrum / CU-PTZ, Erlangen
R26-STAT6VT	Knock-in of a constitutively active form of the transcription factor STAT6 (STAT6vt) in the ubiquitously expressed Rosa26 locus of BALB / c mice. STAT6vt is located behind a loxP- flanked STOP cassette and can only be expressed in the presence of Cre recombinase.	Franz-Penzoldt-Zentrum / CU-PTZ, Erlangen

CD19CreSTAT6VTB6	Mice which express a constitutively active version of STAT6 in B cells	Franz-Penzoldt-Zentrum / CU-PTZ, Erlangen

4. METHODS

4.1 Cellular and immunological methods

4.1.1 Removal of mouse organs and preparation of single cell suspensions

Mice were killed by cervical dislocation under isoflurane anesthesia between 7 and 12 weeks of age.

Spleen and mesenteric lymph nodes

Spleen and mesenteric lymph nodes were removed with sterile forceps without any surrounding fat and placed in 5 mL tubes containing 2 mL RPMI medium. Organs were transferred to a 70 µm cell strainer placed on 50 mL tube. Using a circular motion, spleen and mesenteric lymph nodes were pressed and crushed with the plunger of a 1 mL syringe until only fibrous tissue remained. Clumps in the cell suspension were removed by pipetting the suspension twice through the cell strainer. Single cell suspensions were centrifuged (1200 rpm, 5 min, at 4ºC) and supernatants were discarded. To remove red blood cells, pellets were resuspended in 1 mL ACK lysis buffer for 2 min at room temperature. Cells were washed in RPMI medium, centrifuged and pellets were resuspended in RPMI medium and stored on ice for further analysis.

Bone marrow

Femur and tibia were removed from mice and the muscles were removed from the bones using a sterile paper towel and cleaned bones were placed in a Petri dish. Both ends of each bone were cut off with scissors and the marrow was obtained by flushing out each of the shafts with RPMI medium using a syringe. The marrow was collected in a cell strainer placed on a 50 mL tube. After passing the strainer, the suspension was centrifuged (1200 rpm, 10 min, at 4ºC) and the supernatant was discarded. Red blood cells were lysed with ACK lysis buffer. Cells were washed with RPMI medium, centrifuged and the pellet was resuspended in RPMI medium.

Cell counts

To estimate the number of viable cells in the single cell suspension, trypan blue was used. The dye cannot pass intact cell membranes, but the dead cells absorb the dye and appear blue under the microscope. Cells were prepared for counting using a dilution of 1:20 in trypan blue. 20 µl cell suspension was pipetted into a disposable counting chamber which was inserted into a Cellometer Auto T4 cell counter. Cells were counted automatically.

4.1.2 Flow cytometry and intracellular staining

Surface staining

For staining of surface proteins 1-3x10^6 single cells were washed once in FACS buffer. 100 µL of diluted specific antibodies along with anti-CD16/CD32 blocking antibody (2.4G2) were directly added to the cells, mixed and incubated in the dark at 4ºC for 20 min. Cells were washed in FACS buffer (1200 rpm, 5 min, at 4ºC) to remove unbound antibodies. If biotinylated primary antibodies were used, then cells were stained for an additional 25 min with 100 µL diluted fluorophore-conjugated streptavidin antibodies. Finally, samples were washed and resuspended in 250 µL FACS buffer. Acquisition was performed using a FACS-Canto (FACS Diva software) instrument and analyzed with FlowJo software. Antibodies used in this work are listed in 2.4.

Intracellular staining (Acid treatment)

To 1-3x10^6 single cells 1 mL acid buffer was added and incubated on ice for 2 min. Then, 500 µL of FCS was added to cells, mixed and incubated on ice for further 2 min. Cells were the washed 2 times in FACS buffer. 100 µL of diluted surface antibodies were added to cells and incubated in the dark at 4ºC for 20 min. Cells were washed in FACS buffer (1200 rpm, 5 min, at 4ºC). Subsequently, 300 µL 4% PFA were added to the cells drop by drop and vortexed. Cells were incubated in the dark at room temperature for 20 min and washed in FACS buffer. Supernatants were discarded and 1.5 mL Perm/wash buffer (from Biolegend) were added, centrifuged (1200 rpm, 5 min, at 4ºC). 100 µL of diluted intracellular antibodies in Perm/wash buffer were added to cells and incubated in the dark at room temperature for 20 min. Cells were centrifuged with 1.5 mL Perm/wash buffer. At last, samples were washed in FACS buffer and resuspended in 250 µL FACS buffer.

4.1.3 Cell culture

B cell isolation and stimulation

Primary B cells from WT and STAT6$^{-/-}$ were isolated from the spleen by employing negative selection with the EasySep Mouse B cell isolation kit from stem cell technologies and MojoSort Pan B cell isolation kit from Biolegend. Purified B cells were cultured at a density of 2x10^6 cells/mL in a 24 well plate. Cells were cultured in RPMI medium supplemented with IL-4 (25 ng/mL), anti-CD40 Ab (10 µg/mL) and LPS (2 µg/mL). For further investigation, LPS was replaced with goat anti-mouse IgM-F(ab')$_2$. B-cell cultures were harvested on day 4. If needed fresh medium was added on day 3 and cells used for further experiments.

4.2 Molecular biology methods

4.2.1 SDS-PAGE gel

From the harvested B-cell culture a total of 2x10^6 B cells were centrifuged (4000 rpm, 6 min, at 4ºC). The supernatant was discarded and the cells were washed with ice cold 1x PBS two times (13000 rpm, 6 min, at 4ºC). The cell pellet were lysed with RIPA lysis buffer, the suspension mixed thoroughly by pipetting up-and-down several times and incubated on ice for 20 min. The samples were again centrifuged (13000 rpm, 6 min, at 4ºC) and the supernatants were collected into fresh tubes. Proteins were quantified by BCA assay and 20 µg protein samples were mixed with 6x SDS-PAGE sample buffer. NuPAGE 4–12% Bis-Tris Protein Gels were used to load the protein samples and for gel electrophoresis (at 120 Volts).

4.2.2 Coomassie staining

SDS-PAGE gels were washed several times in water and then fixed in fixation buffer (50% methanol, 2% phosphoric acid) for 30 min. Fixed gels were washed again three times for 10 min each with water and then incubated for 30 min in staining buffer consisting of 34% methanol, 2% phosphoric acid, and 17% ammonium sulfate before adding Coomassie 250 G to stain the gel for 16 h. Before further analysis, the gel was washed several times in water.

4.2.3 Western blot

After SDS-PAGE, the gels were transferred onto Immuno-Blot PVDF membranes using a Trans-Blot Turbo transfer system from Bio-Rad. Membranes were washed and then blocked with

1xTBS + 2%-5% BSA + 0.1% Tween in the dark for 30 min to 1 hour. Membranes were immersed in the solution of primary antibody in 1xTBS + 2%-5% BSA + 0.1% Tween, and incubated in the dark for 1 hour at room temperature or overnight at 4ºC. Then, membranes were washed with 1x TBST three times for 5 min, and subsequently incubated with appropriate secondary antibodies (Donkey Anti-Rabbit IgG coupled to horse radish peroxidase (HRP)) for 1 hour in the dark at room temperature or overnight at 4ºC. After washing the membranes three times for 5 min, signals were visualized using the Signal Fire ECL Western blotting substrate.

For stripping the PVDF membrane, it was incubated in stripping buffer (see table 9: buffers) at room temperature for 2 minutes, followed by 56°C for 15 minutes. Then the blot was washed twice in water and twice in 1X TBST before probing with another primary antibody.

4.2.4 Microarrays

RNA quality was assessed using an Agilent 2100 Bio analyzer platform. A total of 100 ng of each RNA sample was used to generate Cy3-labeled cRNA, which was then hybridized to 8x60K whole mouse genome oligonucleotide microarrays (Agilent Technologies) according to the instructions of the manufacturer. Further analysis was performed in collaboration with Prof. Dr. Diana Dudziak, Laboratory of DC-Biology, Department of Dermatology, University Hospital Erlangen, Friedrich-Alexander-University Erlangen-Nuremberg. The microarray dataset has been submitted to the Gene Expression Omnibus database under accession number GSE84075.

4.2.5 Label-free quantitative LC-MS/MS

After Coomassie staining and several washes (4-5 washes) in water. Gel lanes were cut into 10 slices, were destained and subjected to alkylation followed by in-gel digestion using trypsin, as described (12). The peptide samples were separated on a 50 cm, 75-mm, C18 reversed-phase nano LC column (Acclaim PepMap, 2 mm particle size, 100°A pore size; both from Thermo Scientific) at 40°C using a linear gradient from 3 to 34% (v/v) acetonitrile in 2% (v/v) DMSO and 0.1% formic acid in 30 min and a subsequent increase to 82% acetonitrile in 5 min at a flow rate of 250 nl/min. Peptide mixtures were analyzed by ultra- HPLC tandem MS (MS/MS) using an Ultimate 3000 RSLCnano online coupled to a Q Exactive Plus mass spectrometer (both from Thermo Scientific). Further analysis was performed in collaboration with Prof. Dr. Bettina Warscheid, Faculty of Biology, Department of Biochemistry and Functional Proteomics,

University of Freiburg. MS proteomics data have been submitted to the Proteome Xchange Consortium via the PRIDE partner repository with the dataset identifier PXD005224.

4.2.6 Statistical analyzes

All experiments were repeated at least two times to a maximum of four times (Western blot, FACS analysis) or were measured with 3-4 biological replicates (Microarray, Proteomics) per experimental condition. Unless otherwise indicated, data are represented as mean ± SD, and analysed using Prism software (GraphPad). Proteomics datasets were analysed using Perseus software. Student´s t-test was used for statistical analysis. A *P*-value below 0.05 was considered statistically significant and reported.

5 RESULTS

5.1 Identification of STAT6-regulated proteins in mouse B cells

In order to study the STAT6-regulated genes and proteins, we isolated B cells from wild type (WT) and STAT6 knock out (KO) mice and cultured them *in vitro* for 72 hours. During this time the cells were either left untreated or were stimulated with IL-4. We performed transcriptome and mass spectrometry-based proteome profiling. The transcriptome and proteomic data were already published in Mokada-Gopal et al. J. Immunol. (2017) 198:3737-3745 and used here with permission from the journal.

5.1.1 Identification of IL-4- and STAT6-dependent transcriptomes in primary B cells

The transcription factor STAT6 is phosphorylated within minutes after IL-4R stimulation. Previously, we have shown under the in vivo situation of a helminth infection that B cell-intrinsic STAT6 is essential for germinal-center formation. For the current study, we were interested in IL-4-induced and STAT6-dependent changes in mRNA and protein levels a few days after IL-4 exposure. To identify the IL-4- and STAT6-regulated mRNA transcripts in B cells, we used purified primary mouse B cells from the spleen of naïve WT and STAT6 deficient ($STAT6^{-/-}$) mice on a BALB/c background.

STAT6 deficiency caused no major changes of B cell development; the number and ratio of splenic marginal zone and follicular B cells were comparable between WT and $STAT6^{-/-}$ mice (Figure 5). We set up an in-vitro culture, with purified B cells from three individual WT and $STAT6^{-/-}$ mice and cultured the cells for 4 days in the presence of the LPS and anti-CD40, with or without IL-4 as described in Methods. Anti-CD40 antibodies were used to mimic the signal from Th cells (CD40L) and LPS to increase the number and viability of B cells during the 4-day culture period.

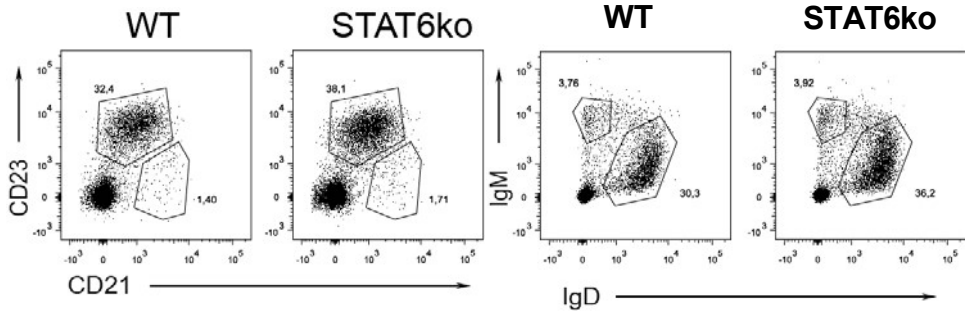


Figure 5: Comparison of B cell development between WT and STAT6$^{-/-}$ mice. Single cell suspensions from the spleens of naïve WT and STAT6ko mice were stained with anti-B220, anti-CD21, anti-CD23, anti-IgD and anti-IgM. Dot plots show the frequencies of follicular B cells (CD23^{hi}CD21^{lo} or IgD^{hi}IgM^{lo}) and marginal zone B cells (CD23^{lo}CD21^{hi} or IgD^{lo}IgM^{hi}).

We further determined the frequency of plasma cells on day 4 of culture and compared WT and STAT6$^{-/-}$ cells. All cultures contained ~96% B cells (B220^{hi}CD138^{-}) and 2% plasma cells (B220^{lo}CD138^{+}) as shown in Figure 6. For microarray analysis (Figure 7), total RNA was isolated from four different cultures (WT or STAT6$^{-/-}$ – IL-4+LPS+anti-CD40 or LPS+anti-CD40).

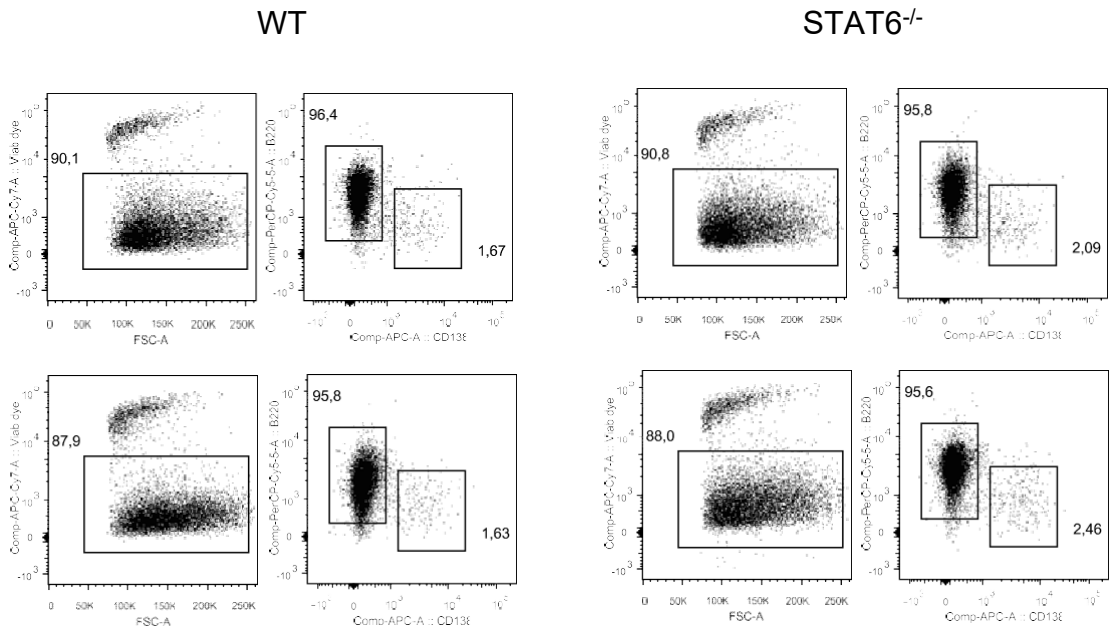


Figure 6: Frequency of plasma cells in WT and STAT6$^{-/-}$
Purified B cells from WT and STAT6-/- stained with anti-B220 (CD45R) and anti-CD138 for plasma cells. Dot plot shows there are were no difference in the frequency of plasma cells between WT and STAT6-/- after 4 days of culture.

5.1.2 The comparison of gene expression profiles between WT and STAT6-/-

5.1.2.1 Identification of IL-4– and STAT6-regulated mRNA transcripts in primary mouse B cells

To characterize the IL-4- and STAT6-regulated transcriptome in B cells, we first purified B cells from three individual WT or STAT6KO mice and cultured them for 4 days in vitro with medium containing LPS and anti-CD40 in the presence or absence of IL-4 as described in Methods. Total RNA was isolated from these 4 different cultures and subjected to microarray analysis (Figure 7). The comparison of gene expression profiles between unstimulated WT and STAT6ko samples or between IL-4-stimulated STAT6ko and unstimulated STAT6ko samples revealed that only about 20 mRNAs were more than 3-fold differentially expressed (Figure 8A, bottom). However, 462 mRNAs were more than 3-fold up- or down-regulated by IL-4 in B cells from WT mice and similar numbers were observed for the comparison of IL-4-stimulated WT vs IL-4-stimulated STAT6ko samples (Figure 8A, top). To further investigate whether the IL-4-regulated genes in WT B cells are dependent on STAT6 signaling we compared the differentially expressed gene sets between ‘WT+IL-4 vs WT’ and ‘WT+IL-4 vs STAT6ko+IL-4’ samples. The more than 3-fold IL-4-regulated genes were indeed largely dependent on STAT6 signaling (290 of 462 genes) (Figure 8B). Next, we performed unsupervised hierarchical cluster analysis of 1000 most differentially expressed genes among all 12 samples, which comprise three replicas each for the four conditions described above. The three ‘WT+IL- 4’ replicate samples showed a gene expression profile that was very distinct from all other samples (Figure 8C). This could also be visualized by principal component analysis (Figure 8D). These findings illustrate that the IL-4-regulated transcriptome in primary B cells is largely dependent on STAT6 signaling.

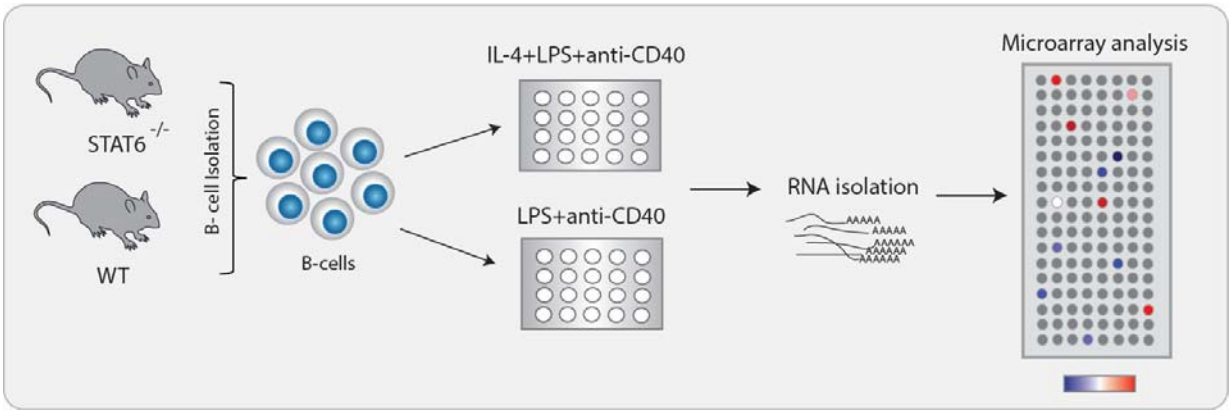
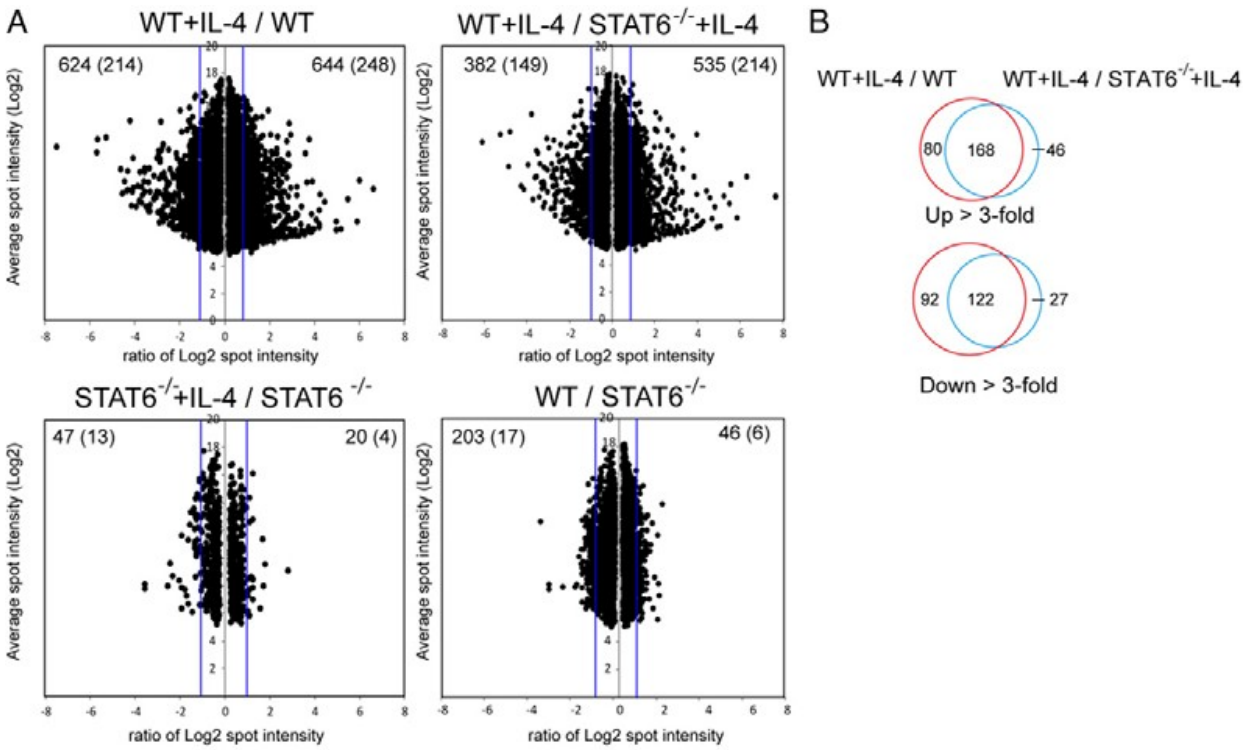


Figure 7: Microarray analysis setup. B cells from both the strains where stimulated with LPS+anti-CD40 with or without IL-4 as mentioned in the materials and methods. Total RNA was isolated from these samples and subjected to microarray analysis.



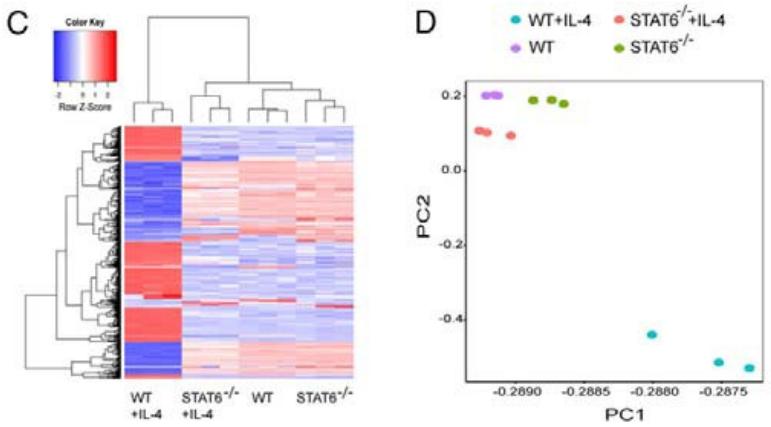


Figure 8: Microarray analysis of STAT6-regulated genes in B cells. (A) Dot plots show pair-wise comparisons of the transcriptomes from splenic B cells of wild-type (WT) and STAT6ko mice cultured for four days in vitro with LPS and anti-CD40 in the presence or absence of IL-4. The plots illustrate the ratio of Log_2 spot intensity (x axis) and average spot intensity Log_2 (y axis) based on the average values from three biological replicates per condition and a p-value of <0.05. The blue lines indicate the cut-off for transcripts that were at least 2-fold differentially expressed. The numbers indicate the number of transcripts that were 2-fold (no brackets) or 3-fold (in brackets) differentially expressed. The Venn diagrams show the number of transcripts that were >3-fold up- or down-regulated by IL-4 in the presence or absence of STAT6. The overlap contains transcripts that were regulated by IL-4 in WT B cells in a STAT6-dependent manner. **(B)** The 1000 most differentially expressed genes among all samples were subjected to hierarchical cluster analysis. Red indicates up-regulated and blue indicates down-regulated mRNA transcripts. **(C)** Principle component analysis for microarray data showing one colored dot for each sample. The data are published in Mokada-Gopal et al. J. Immunol. (2017) 198:3737-3745 and used here with permission from the journal.

5.1.2.2 Proteome analysis of IL-4-stimulated WT and STAT6-deficient B cells.

Since mRNAs are regulated by several post-transcriptional events that modulate their stability it is difficult to predict differences at the protein level from transcriptome data. Furthermore, the turnover of proteins is also tightly regulated, which further complicates reliable predictions. Therefore, we investigated the IL-4-induced and STAT6-dependent proteome in B cells by label-free quantitative liquid chromatography (LC) and mass spectrometry (MS) analysis as described in Methods.

Here we used 4-5 biological replicates from 4 different B cell cultures described in Figure 9A. In total, we could identify 5865 distinct proteins (Figure 9B) with one sample showing bad correlation due to technical reasons (Figure 9C). Hence, this sample was removed for further downstream analysis to avoid bias. We found that 252 proteins were up- or downregulated more than 2-fold by IL-4 stimulation in WT B cells (Figure 10A, top).

Restricting the data set to more than 3-fold regulated proteins reduced the number to 115 (59 up and 56 down). The analysis of the IL-4-regulated and STAT6-dependent proteome (comparison of 'WT+IL-4 vs STAT6ko+IL-4') revealed that 174 proteins were differentially expressed more than 2-fold and 75 proteins were up-regulated and 74 down-regulated more than 3-fold (Figure 10A, top). IL-4 induced also some changes in the proteome of B cells from STAT6ko mice but almost no differences were observed by comparing the proteomes of WT and STAT6ko B cells that had not been cultured in the presence of IL-4 (Figure 10A, bottom). The majority of more than 3-fold IL-4-regulated proteins in WT B cells were STAT6-dependent (75 of 115) as shown by the overlap of protein sets in Figure 10B. However, we also found 75 proteins that were more than 3-fold differentially expressed between 'WT+IL-4 vs STAT6ko+IL-4' but not between 'WT+IL-4 vs WT'.

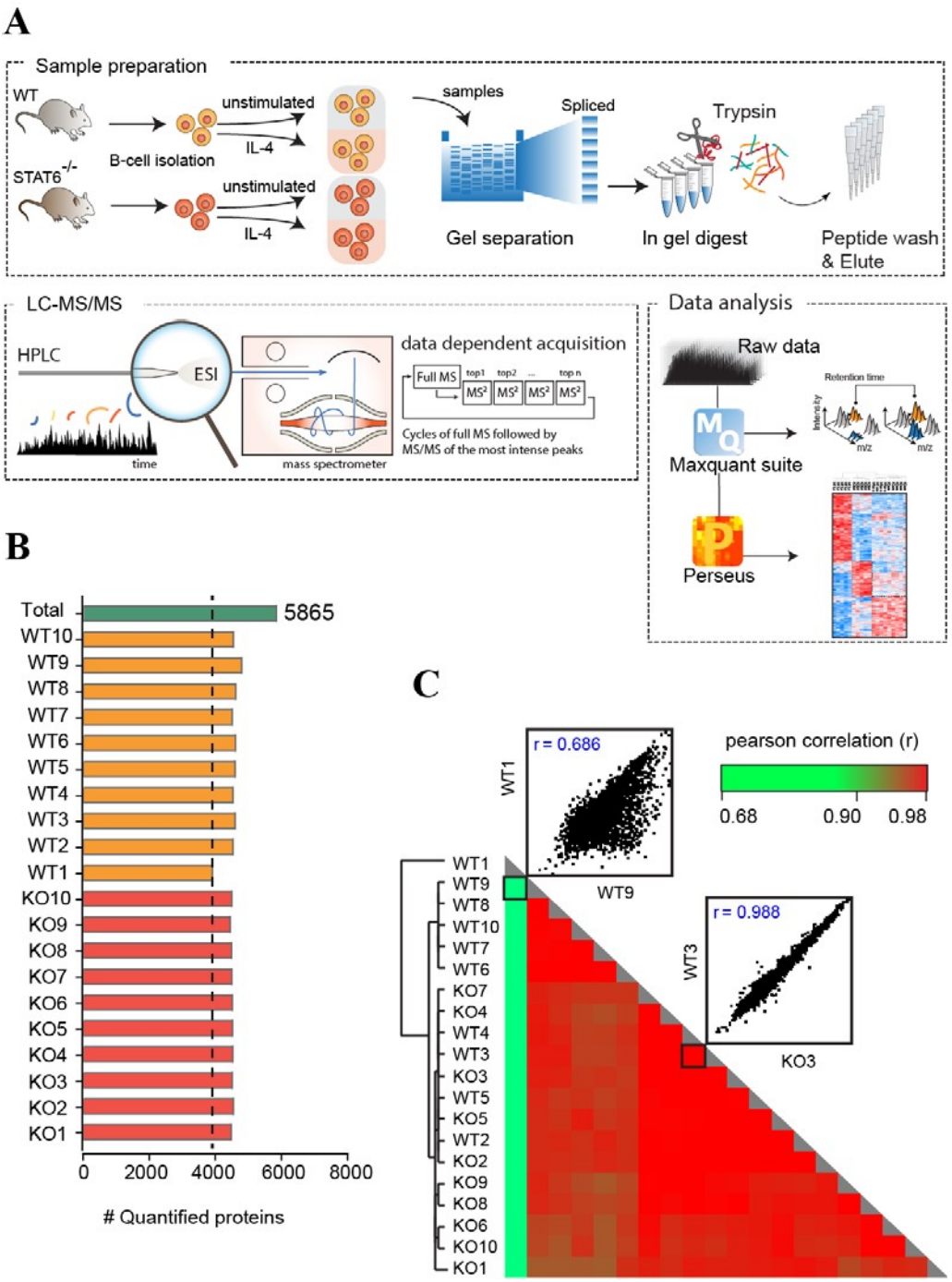


Figure 9: A, schematic of B-cell proteome analysis. Following B-cell collection from mouse, the cells were stimulated with IL-4 or without and lysed in lysis buffer. The lysates were SDS-PAGE gel separated and spliced into ten fragments. The gels were digested with Trypsin and peptides were eluted and analyzed in LC-MS/MS. The MS raw files were analyzed in Maxquant and Perseus. **B,** Summary of quantified proteome in total and per sample. Sample WT1 shows the

lowest quantified proteome in numbers. **C,** Heatmap of sample correlation matrix of all the measured samples based on Pearson correlation values. The reproducibility between the samples are highlighted. WT1 sample shows the least correlation and reproducibility with all the samples in this study.

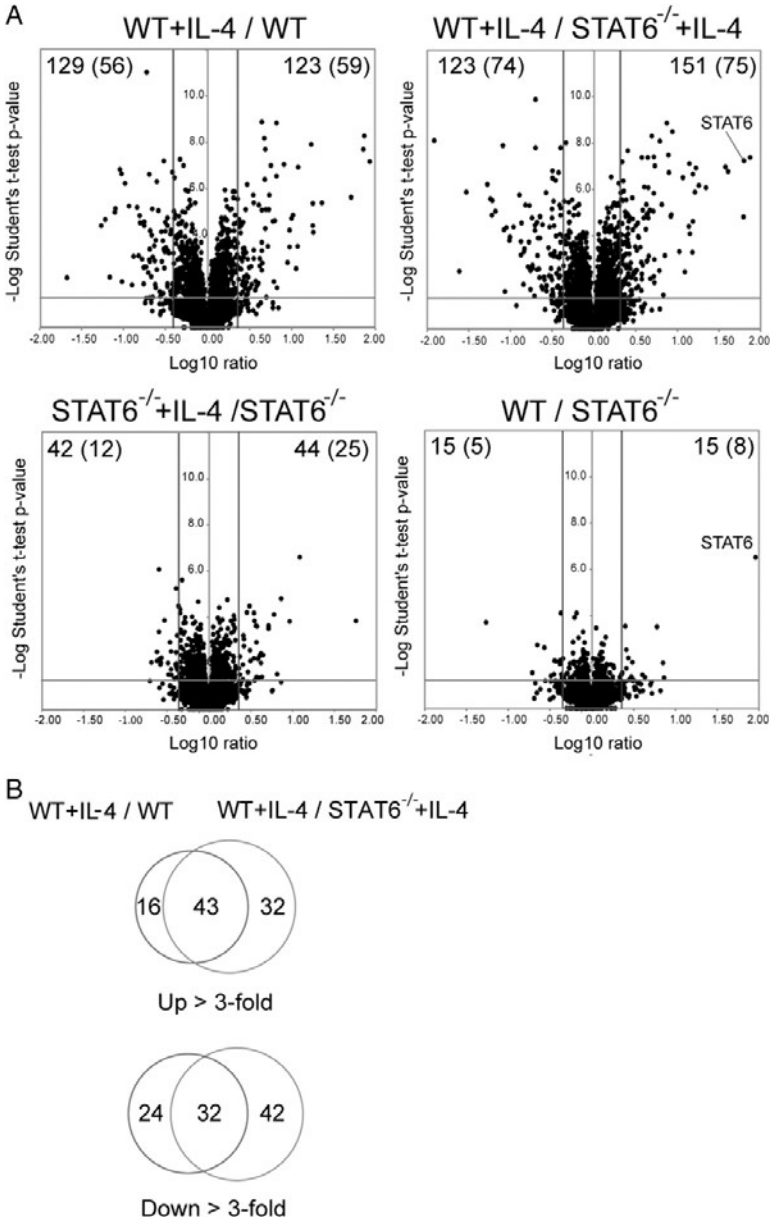


Figure 10: Label-free quantitative mass spectrometry to identify IL-4/STAT6-regulated proteins in B cells. (A) Volcano plots show the Log_{10} ratio (x-axis; indicates the difference in expression) plotted against the -Log_2 *p*-value (y-axis; indicates the reliability of the values) for

the comparison of indicated cultures with 3 biological replicates per condition. The horizontal line is the cut-off for $p<0.05$. The vertical lines are the cut-off for proteins that were at least 2-fold differentially expressed. (B) The Venn diagrams show the number of proteins that were >3-fold up- or down- regulated by IL-4 in the presence or absence of STAT6. The overlap contains proteins that were regulated by IL-4 in WT B cells in a STAT6-dependent manner. The data are published in Mokada-Gopal et al. J. Immunol. (2017) 198:3737-3745 and used here with permission from the journal.

5.2 STAT6 dependent protein and gene expression

5.2.1 Correlation between proteomics and transcriptome data

To further investigate whether the STAT6-dependent proteins that were up- or downregulated ≥3-fold are also regulated at the mRNA level we correlated the microarray data with the proteome data. To our knowledge, such a direct comparison of STAT6-dependent gene expression at the mRNA and protein levels has not been described before. The added value of this comparison is reflected by the ability to determine whether changes in protein levels are based on changes in transcription or are due to posttranscriptional mechanisms that regulate protein turnover. This information can be important, for example, for potential therapeutic interventions aiming at lowering protein levels by targeting specific mRNAs. Differential expression of essentially all proteins for which a correlation could be identified in the microarray data set showed that the expression was regulated in the same direction at the transcriptional level (Figure 11). This indicates that posttranscriptional and posttranslational mechanisms play a minor role in the IL-4–regulated proteome in B cells during the 4-d timeframe of stimulation used in this experimental setup. The list of IL-4–induced and STAT6-dependent proteins that were regulated ≥3-fold includes components of the MHC class II complex (H2-DM), cytokine receptor signaling (IL-5Ra, Socs2, STAT5), BCR complex (CD79b), and surface receptors (Tnfrsf8 [also known as CD30], Ly75 [DEC205], and others) (Figure 11). Interestingly, the classical T cell marker Thy1 (CD90) appeared to be the most strongly IL-4–induced STAT6-dependent protein (76- fold upregulation at the protein level and 249-fold upregulation at the mRNA level). The signaling adaptor Lcp2 (SLP-76) is another well-characterized T cell–associated protein that we identified to be upregulated >40-fold by IL-4 in B cells. The list of IL-4-suppressed and STAT6-dependent

proteins includes the inhibitory receptors CD72 and FcRL1, many GTP-binding proteins, Irf9, STAT4, and the tyrosine kinase Syk, which is known to be important for signaling through the BCR (Figure 11).

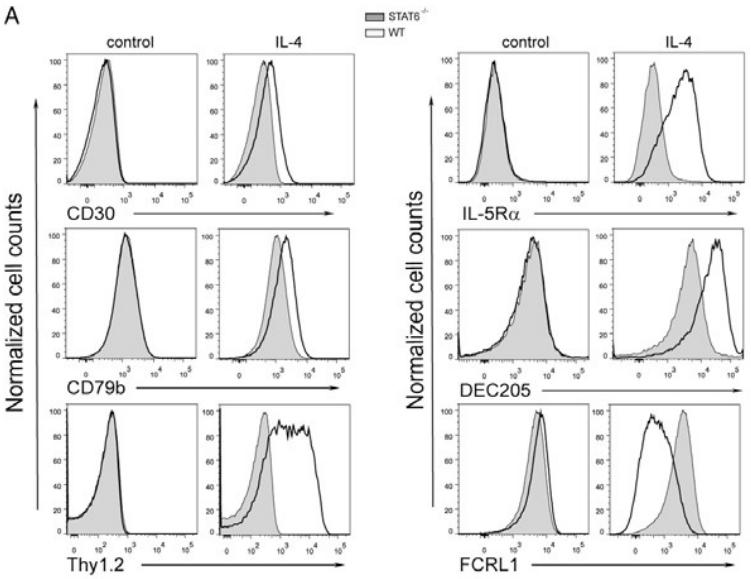
gene symbol	protein FC	mRNA FC
Agfg2	3,05	nf
Ahcyl1	12,44	nf
Arid5a	4,27	2,43
Ass1	4,38	1,71
Birc2	3,19	1,23
Camk1d	3,46	2,71
Casp6	38,58	6,45
Cd79b	6,92	1,79
Ckap4	6,25	1,16
Clcn3/5	8,49	1,54
Cnrip1	3,82	3,23
Cpox	4,50	6,77
Cradd	16,53	4,20
Dnaaf2	4,44	4,08
Dock10	14,22	1,92
Efhd2	5,42	4,79
Eftud1	4,07	2,46
Epha2	5,38	nf
Fads1	3,28	nf
Fam118b	3,21	1,74
Ftl1/2	3,67	1,71
Galk2	7,61	5,86
Gatm	7,98	1,96
Gnb4	14,34	23,75
H2-DMa	3,81	2,79
H2-DMb2	4,76	1,97
Hspa1a/1b	5,23	nf
Ido1	5,38	0,82
Igf2r	3,90	1,80
Ighv1-31+others	4,79	nf
Igkv17-127/-121	3,31	nf
Il4i1	4,34	1,97
Il5ra	22,39	78,79
Impdh1	6,19	nf
Itpk1	3,17	1,52
Kidins220	3,83	nf
Kmo	4,62	2,68
Lcp2 /SLP-76	41,36	4,59
Lmo7	15,33	3,05
Ly75 /DEC205	6,67	3,66
Nedd1	3,00	1,20
Nfkbia	3,06	0,47
Pcyox1l	4,52	1,95
Plbd1	3,92	1,92
Plcd1	16,92	28,05
Prex1	3,23	1,57
Prkca	11,85	2,60
Psmb6	7,17	1,25
Pxk	5,28	1,61
Rab35	7,97	6,96
Ripk3	4,02	1,95
Samsn1	5,00	3,10
Selenbp1/2	8,59	nf
Slc4a7	15,64	3,97
Snx8	4,52	2,31
Socs2	4,58	38,05
St6gal1	3,50	1,99
Stat5a	4,32	2,50
Stat5b	5,30	1,75
Stat6	63,74	3,56
Tes	3,42	1,59
Thy1	76,24	238,86
Tmed8	13,96	5,58
Tmem176b	15,72	6,15
Tnfrsf8 /CD30	18,45	5,31
Tnip2	3,30	1,18
Trp53i11	3,74	1,38
Trpv2	5,29	2,57
Twsg1	3,13	2,79
Ubl3	8,86	2,57
Vim	3,18	1,97
Znf608	63,72	nf
MLV-related	10,71	nf
Ig-kappa-V-region	3,73	nf
Ig-kappa-V-region	3,46	nf

gene symbol	protein FC	mRNA FC
Actb	-40,42	nf
Adap1	-3,11	-1,49
Ahnak	-5,66	nf
Akap13	-3,07	nf
Anxa1	-10,20	-5,66
Bst2	-3,26	-1,67
Ca2	-7,71	nf
Ccdc50	-5,46	-2,60
Ccr7	-7,42	nf
Cd72	-3,02	-1,46
Cd80	-4,76	-5,58
Cmpk2	-4,62	-1,44
Cyb561a3	-3,23	-1,55
Cybb	-3,09	-1,93
Dhx58	-5,82	-2,46
Ehhadh	-3,03	nf
Eif2ak2	-3,69	-1,24
Fam20b	-4,47	nf
Fam49a	-5,04	-2,38
Fcrl1	-12,15	-3,41
Fgfr1op	-3,13	-1,52
Fscn1	-6,52	-4,50
Fyco1	-3,65	-1,49
Gbp2	-5,04	-3,56
Gbp4	-4,37	-1,16
Gbp5	-3,14	-2,89
Gbp6/10	-11,89	-1,84
Gbp7	-4,39	-1,93
Gtse1	-3,86	nf
Hnrnph3	-3,15	nf
Ifi44	-80,87	-6,36
Ifi44l	-33,50	nf
Ifih1	-6,83	-3,32
Ifit1	-16,29	-6,11
Ifitm3	-15,02	-2,00
Igtp	-2,99	-1,54
Iigp1	-11,47	nf
Irf9	-5,41	-1,85
Irgm1	-4,07	-3,61
Isg15	-4,92	-2,97
Kctd12	-3,46	-2,75
Ksr1	-5,11	-2,00
Lacc1	-4,44	-2,69
Lactb	-3,41	-2,48
Ly6a	-7,43	-3,76
Ly6c1/2	-4,22	-3,78
Ly6d	-9,98	-3,12
Map3k5	-4,10	-1,65
Mpeg1	-6,91	-9,19
Ms4a4c	-18,74	-1,84
Nmd3	-3,18	nf
Nt5c3a	-4,74	nf
Oas1a/g	-17,17	-2,50
Oas3	-9,17	-2,11
Oasl1	-5,91	-3,48
Pls1	-5,66	nf
Pms1	-3,66	-1,35
Pou2f2	-4,35	-3,03
Pstpip1	-3,23	-1,65
Pyhin1	-6,73	-2,27
Rab30	-5,36	-3,20
Rasgrp3	-3,68	-3,73
Rassf4	-3,37	-1,77
Rnf213	-3,13	-1,29
Rnpep	-7,26	-2,36
S100a10	-4,66	-3,73
Sco2	-3,89	-0,86
Snx20	-6,06	-1,20
Stat4	-3,05	nf
Stk39	-2,99	-3,92
Syk	-4,92	-4,29
Synj2bp	-3,03	-1,51
Tgtp1	-16,52	-6,06
Zbp1	-11,00	-1,77

Figure 11: List of most differently expressed STAT6-regulated proteins and their correlation to mRNA transcript levels. The list shows, in alphabetic order, fold changes (FC) of IL-4-induced (left) or –repressed (right) and STAT6-regulated proteins in comparison to FC in corresponding mRNA transcripts of datasets from WT+IL-4 and STAT6ko+IL-4 B cells. Differences at the protein level are indicated in red/dark green (up/down >10-fold), orange/light green (up/down>5-fold), yellow/olive (up/down >3-fold). Differences at the mRNA transcript level are indicated in red/dark green (up/down >4-fold), orange/light green (up/down>2-fold), yellow/olive (up/down>1.5-fold). The data are published in Mokada-Gopal et al. J. Immunol. (2017) 198:3737-3745 and used here with permission from the journal.

5.2.2 Validation of STAT6 dependent proteins – FACS and Western blot

Several up- and down regulated proteins were selected from the list in Figure 11 and confirmed by flow cytometry and western blot analysis. Purified primary B cells from spleen of WT and STAT6$^{-/-}$ mice were cultured for 4 days in medium containing anti-CD40 and LPS or anti-IgM F(ab')$_2$ in the presence or absence of IL-4. STAT6-dependent expression of CD30, CD79b, Thy1.2, IL-5Ra, DEC205 and FCLR1 was confirmed by flow cytometry (Figure 12). Western blot analysis confirmed the IL-4-induced and STAT6-dependent upregulation of STAT5a/b and SLP-76, and downregulation of proteins such as Syk by IL-4 in B cells from WT mice but not in STAT6$^{-/-}$ mice (Figure 13).



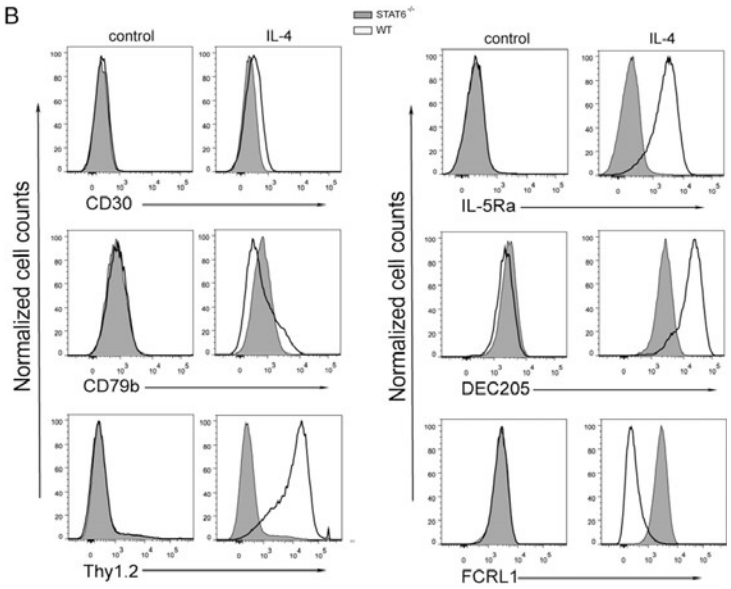


Figure 12: Confirmation of STAT6-dependent proteins by flow cytometry. **A-B)**. Flow cytometry analysis for selected STAT6-regulated proteins in WT and STAT6$^{-/-}$ B cells cultured for 4 day with LPS and anti-CD40 (A) or anti-IgM–F(ab')$_2$ and anti-CD40 (B) in the presence or absence (control) of IL-4. Line graph overlays show the expression level of indicated surface markers on B cell cultures from WT or STAT6$^{-/-}$ mice. The results are representative of three **(A)** and two **(B)** independent experiments. The data are published in Mokada-Gopal et al. J. Immunol. (2017) 198:3737-3745 and used here with permission from the journal.

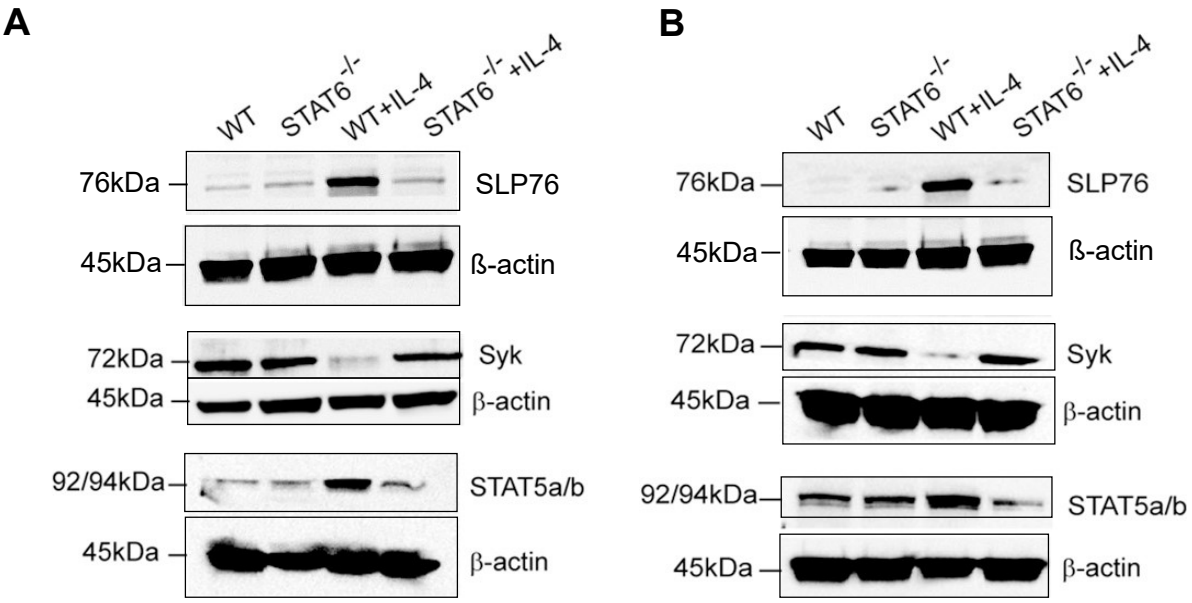


Figure 13: Confirmation of STAT6-dependent proteins by Western blot. Western blot analysis shows the expression of SLP-76, Syk, and STAT5a/b in B cells from WT and STAT6$^{-/-}$ mice cultured for 4 d with LPS and anti-CD40 (A) or anti-IgM–F(ab)$_2$ and anti-CD40 (B) in the presence (IL-4) or absence of IL-4. β-actin was used as loading control. The data are published in Mokada-Gopal et al. J. Immunol. (2017) 198:3737-3745 and used here with permission from the journal.

5.3 Role of STAT6 in GC formation

5.3.1 STAT6 influences the migratory behavior of B cells within the spleen

Our group has previously shown that in mixed bone marrow chimeras the GC formation was dependent on STAT6 expression in B cells. We now liked to determine how STAT6 influences the migratory behavior of B cells within the spleen. We studied the role of STAT6 for B cell entry into and localization within the GC by two main approaches. First, naïve splenic B cells were transferred from WT (CD45.2) into Sheep Red Blood Cell (SRBC)-immunized WT (CD45.1) mice on day 8 after immunization and analysis on day 12 (Figure 14).

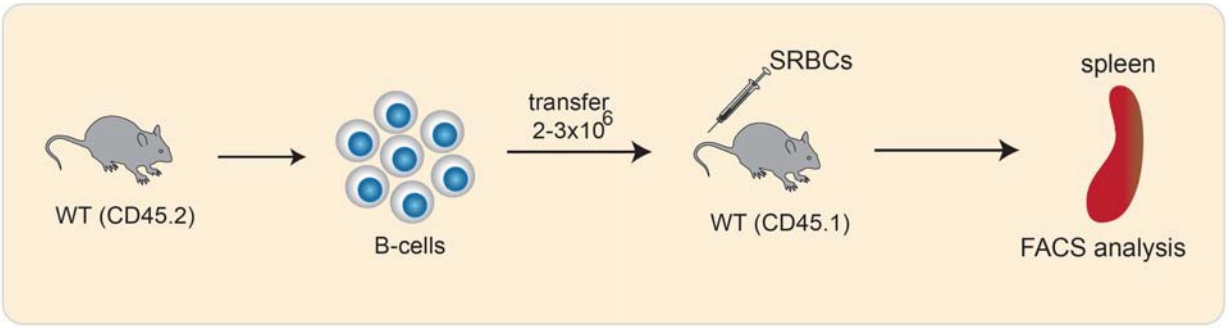


Figure 14: Experimental setup – transfer of **naïve** WT B cells (CD45.2) Balb/c into Sheep Red Blood Cells (SRBCs) immunized WT (CD45.1) Balb/c on day 8. FACS analysis was done on day 12.

We observed that transferred naïve WT B cells (CD45.2) contributed to germinal center formation under these conditions. Analysis was done by staining for CD38 (non-GC) and GL-7 (GC) as shown in the FACS plot (Figure 15).

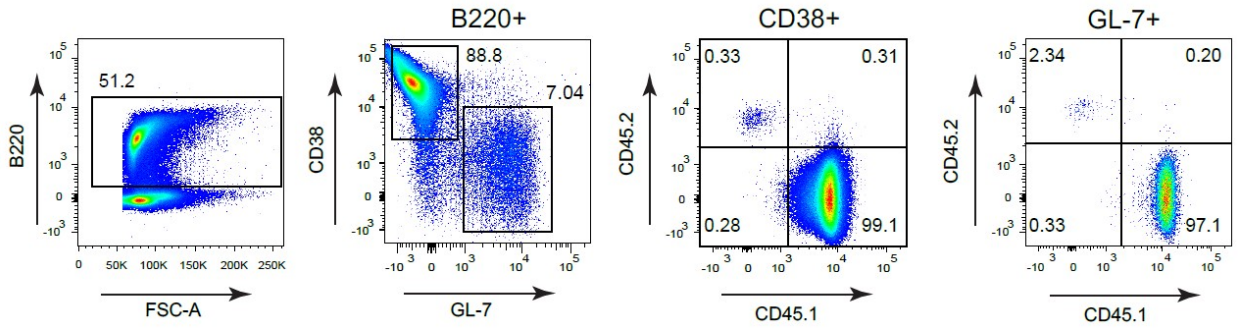


Figure 15: Transfer of naïve B cells WT (CD45.2) into SRBCs immunized WT (CD45.1) mice on day 8. FACS plots show a representative gating strategy of germinal center B cells from SRBCs immunized mice on day 12 after immunization. Germinal center B cells were identified as $CD38^{-}/GL\text{-}7^{+}$ and stained for CD45.1 and CD45.2 to distinguish transferred B cells (CD45.2+) from recipient B cells (CD45.1+). The above experiment was repeated more than three times with 3-5 mice per group. Above plots shows the constitutive result.

In the next step we activated the WT B cells (CD45.2) with IL-4 and LPS, four days prior to the transfer into SRBC immunized WT mice (CD45.1) mice and analyzed them on day 12 (Figure 16). We observed that even after the activation of WT (CD45.2) B cells with IL-4 and LPS for four days, B cells were able to contribute to germinal center formation (Figure 17 A-B).

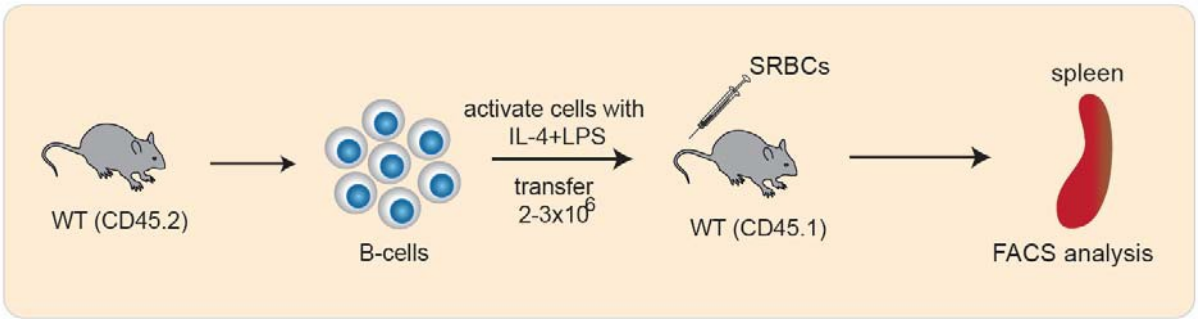


Figure 16: Experimental setup – transfer of **activated** WT B cells (CD45.2) Balb/c into Sheep Red Blood Cells (SRBCs) immunized WT (CD45.1) Balb/c mice on day 8 .

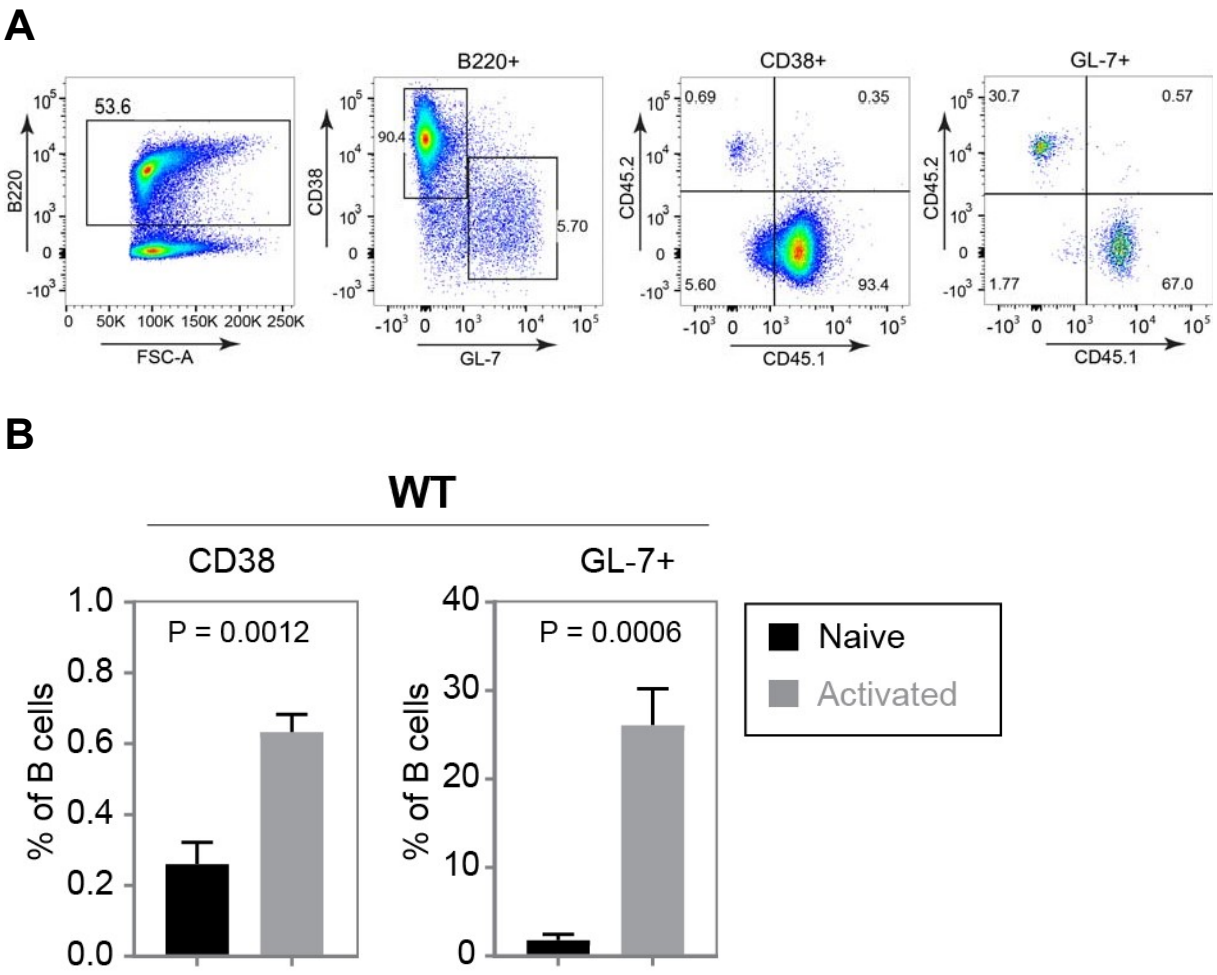


Figure 17: Activated WT (CD45.2) B cells into SRBCs WT (CD45.1) mice. A. FACS plots show a representative gating strategy of germinal center B cells from SRBCs immunized mice on day 12 after immunization. Germinal center B cells were identified as $CD38^{-}/GL\text{-}7^{+}$ and stained for CD45.1 and CD45.2 to distinguish transferred B cells (CD45.2+) from recipient B cells (CD45.1+). **B.** Bar graphs show frequency (%) of CD38 and GL-7 from naïve and activated WT B-cells. Data represent means ± SD of 3-4 independent experiments.

5.3.2 Germinal center formation is dependent on STAT6

We further performed the same experiments with B cells from STAT6$^{-/-}$ mice (Figure 18). Transferred B cells (CD45.2) did not migrate onto germinal center population whereas in the CD38+ population only few transferred were seen on day 12.

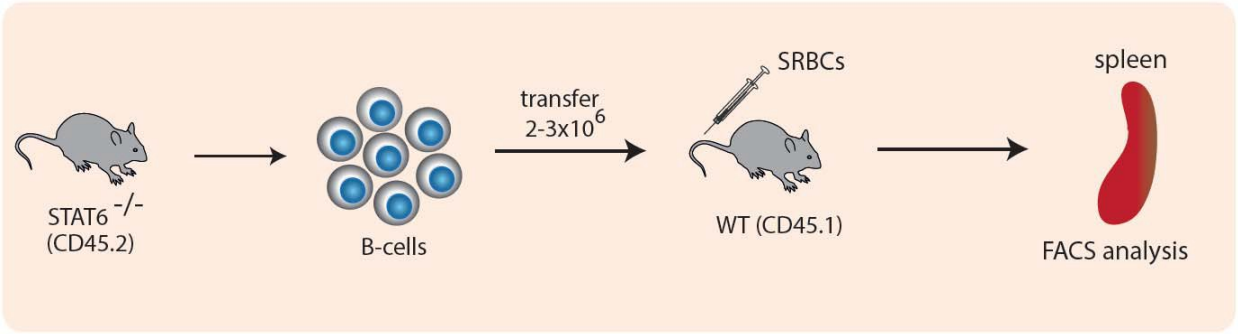


Figure 18: Experimental setup – transfer of **naïve** STAT6$^{-/-}$ (CD45.2) B cells Balb/c into Sheep Red Blood Cells (SRBCs) immunized WT (CD45.1) Balb/c mice on day 8.

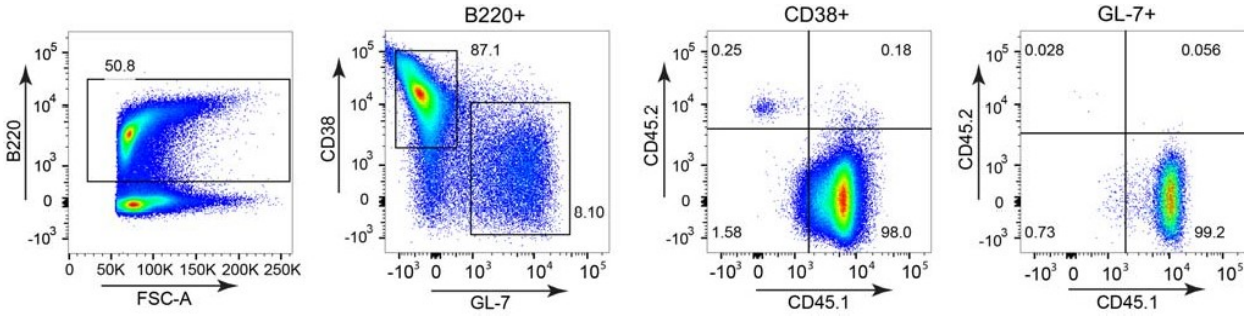


Figure 19: transfer naïve STAT6$^{-/-}$ (CD45.2) B cells into SRBCs WT (CD45.1) mice. The above plots shows that transferred naïve CD45.2 is located in CD38+ population where absent in the GL-7+ population on Day 12 after SRBC immunization.

Above FACS plot (Figure 19) represents results obtained by transfer of naïve STAT6$^{-/-}$ (CD45.2) B cells into SRBCs-immunized WT mice. We observed that the transferred CD45.2 B cells were only present in the non-germinal center population (CD38+). This result confirms that the germinal center formation is dependent on STAT6 in B cells. To further analyze GC recruitment of activated STAT6$^{-/-}$ (CD45.2) B cells, they were stimulated with IL-4 and LPS 4 days before the

transfer into SRBCs-immunized WT (CD45.1) mice (Figure 20) on day 8 after immunization. The frequency of transferred cells and recipient cells was analyzed within the populations of non-GC B cells (CD38+) and GC B cells (GL-7+).

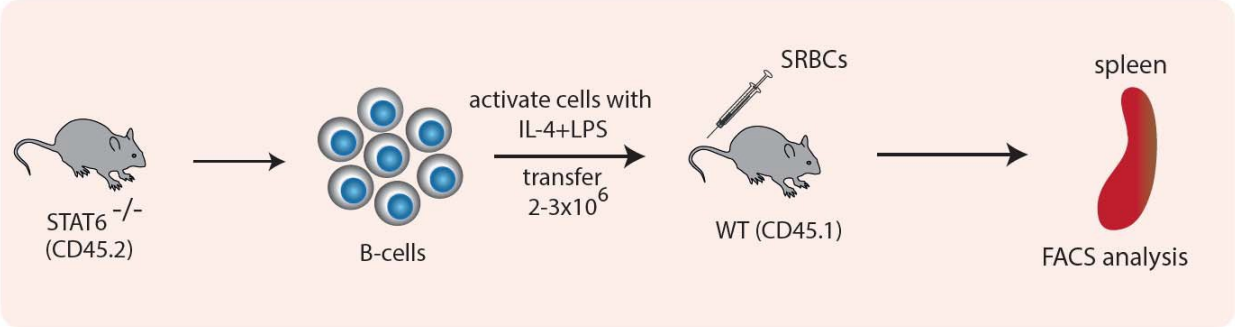
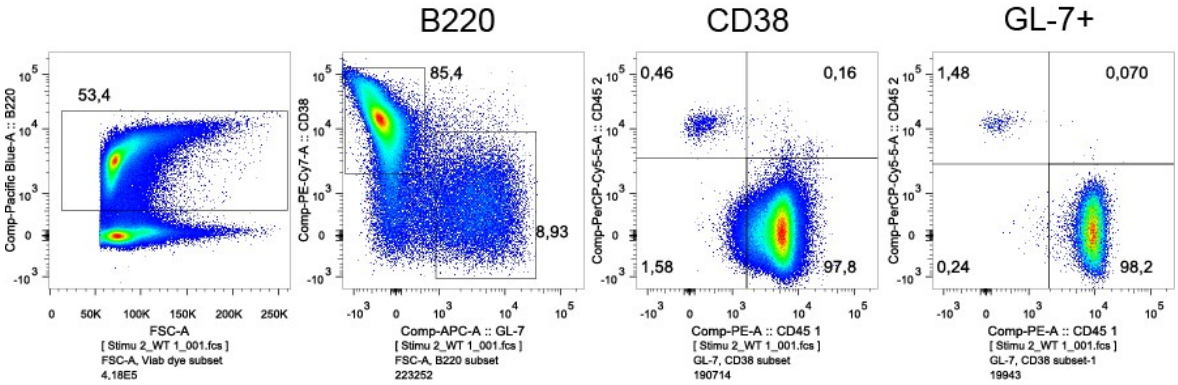


Figure 20: Experimental setup – transfer of **activated** STAT6$^{-/-}$ (CD45.2) B cells into WT (CD45.1) Balb/c mice immunized with SheepRed Blood Cells (SRBCs) on day 8 after immunization.

A



B

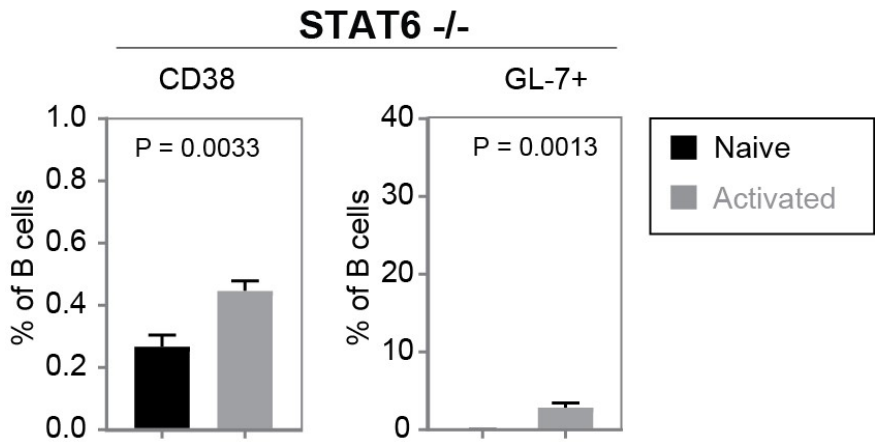


Figure 21: Transfer of activated STAT6$^{-/-}$ (CD45.2) B cells into SRBCs-immunized WT (CD45.1) mice. A. FACS plots showing the representative gating strategy of germinal center B cells from SRBCs-immunized mice on day 12 after immunization. Germinal center B cells were identified as CD38^{-}/GL-7^{+} and stained for CD45.1 and CD45.2 to distinguish transferred B cells (CD45.2+) from recipient B cells (CD45.1+). **B**. Bar graphs show the frequency (%) of CD38 and GL-7 from naïve and activated STAT6 KO B-cells. Data represent means ± SD of 3-4 independent experiments.

When we transferred the activated STAT6$^{-/-}$ (CD45.2) B cells into immunized WT (CD45.1) mice, we noticed to our surprise that transferred CD45.2 B cells showed expression of the germinal center marker GL-7 but they also expressed low levels of CD38 (Figure 21A-B).

5.3.3 Recruitment into existing GCs requires antigen-specificity

To address whether all transferred naïve B cells from WT mice migrated into the germinal center irrespective of their antigen-specificity we used B cells from swHEL mice that are specific for hen-egg lysozyme and, therefore are not activated after transfer in SRBCs-immunized mice (Figure 22). We assumed that transferred swHEL B cells should only be found within the population of non-GC B cells.

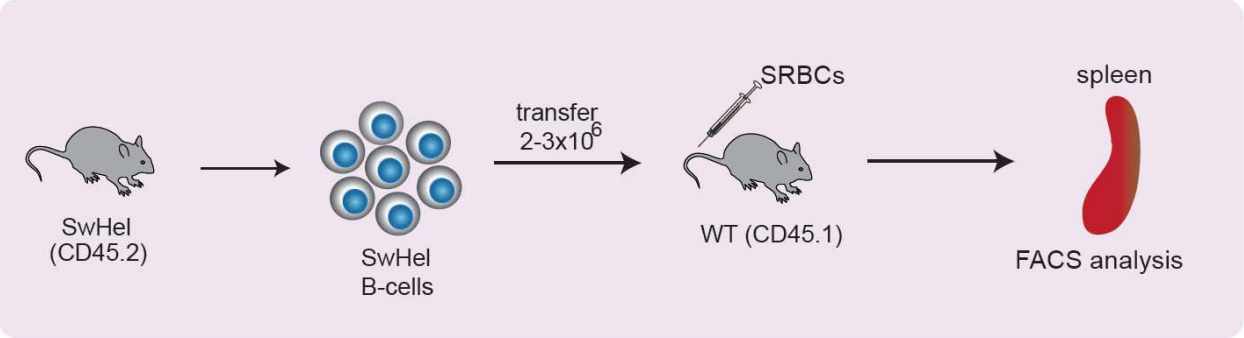


Figure 22: Overview of transfer experiment with SwHEL B-cells into SRBCs-immunized mice. Isolated B cells from SwHEL (CD45.2) mice were transferred into SRBCs-immunized WT (CD45.1) mice on day 8 after immunization. Four days after cell transfer, on day 12 after immunization, spleens were isolated from immunized mice and analyzed.

Transferred SwHEL B cells and recipitent WT mice were stained for non-germinal center (CD38) and germinal center (GL-7) B cells, and for CD45.1 and CD45.2 to distinguish wild type and SwHEL B cells. We could clearly distinguish populations of CD38 and GL-7 wild type (CD45.1) and SwHEL (CD45.2) B cells.

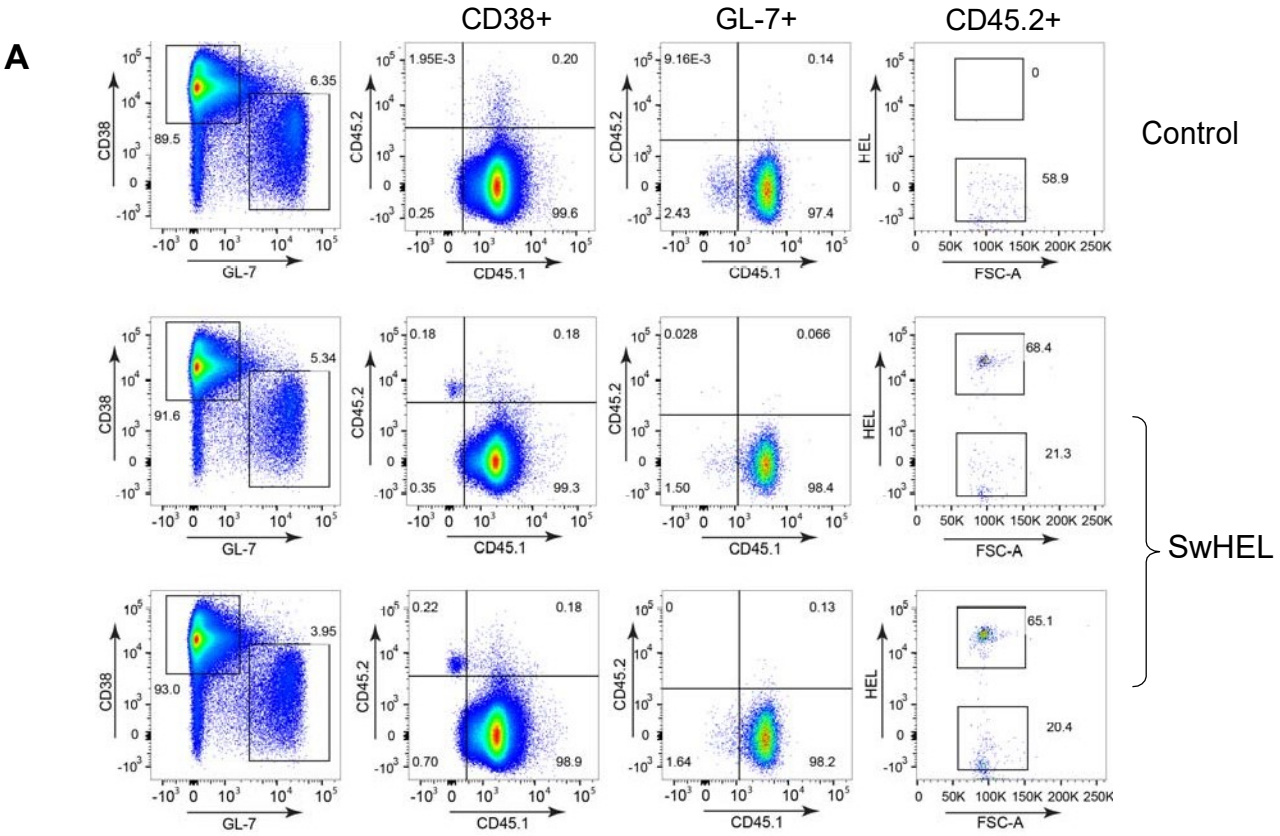


Figure 23: FACS analysis of GC B cells induced by sheep red blood cell immunization. A). WT (CD45.1) mice were immunized i.v. with 2-3 x 10^6 SRBCs. 8 days post-immunization SwHEL B cells were transferred into two mice. 12 days post-immunization, the mice were sacrificed and GC B cells detected by flow cytometry. This experiment was performed for 4 times and 2-4 mice were used per group. FACS plots are pre-gated on B220+ cells and show expression of CD38 versus GL-7, the frequency of CD45.2 and CD45.1 cells within the non-GC gate (CD38+) and GC gate (GL-7+), and the Expression of the HEL-BCR on CD45.2+ cells (right) .**B).** Bar graphs shows frequency of CD38, GL-7 and HEL positive B-cells in control vs SwHEL mice. Data represent means ± SD of 2-4 independent experiments.

5.4 Characterization of CD19Cre_STAT6VT mice

5.4.1 Generation of CD19Cre_STAT6VT mice

Former members of the Voehringer lab generated Rosa26-STAT6vt knock-in mice. To obtain these mice, a constitutively active version of STAT6 was inserted into a Rosa26 targeting vector and BALB/c ES cells were used to generate Rosa26-STAT6vt mice. The STAT6vt molecule contains a VT=>AA mutations next to Tyr641. This causes spontaneous Tyr641 phosphorylation by an IL- 4-independent and unknown kinase. The Rosa26-STAT6vt mice were crossed to CD19Cre-mice to create a mouse strain in which constitutively active STAT6 is selectively expressed in B cells (Figure 24). These CD19Cre_STAT6vt mice will be used in the current study to further characterize the role of STAT6 in different B cell subsets, GC formation, PC differentiation and survival of memory B cells.

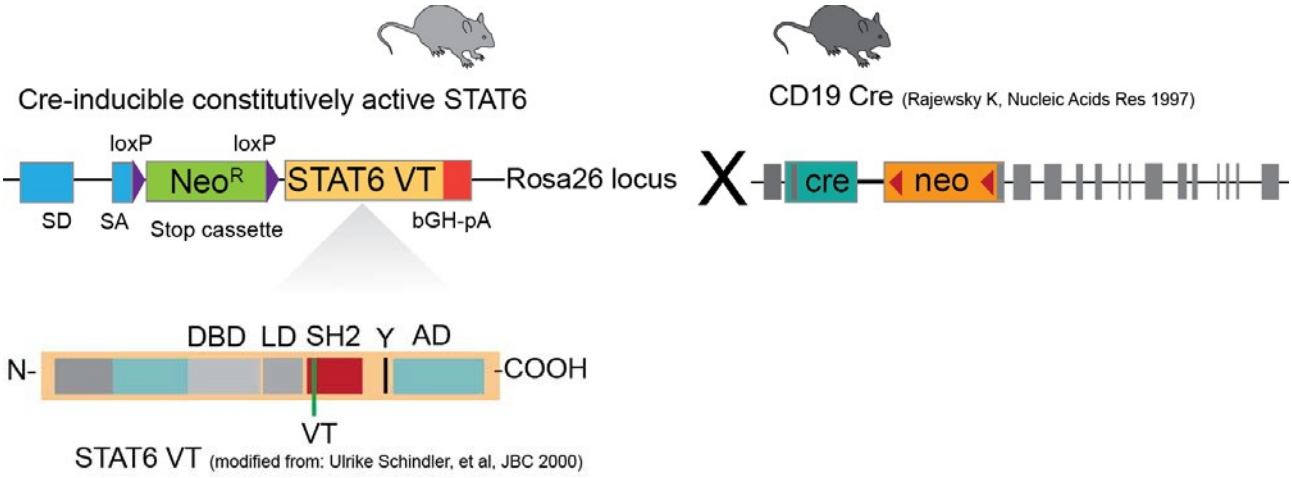


Figure 24: Generation of CD19Cre_STAT6VT mice. A constitutively active version of STAT6 (STAT6vt) had been previously generated by alanine scanning mutagenesis of the SH2 domain (98). The exchange of Val 547 and Thr 548 to Ala resulted in spontaneous Jak1/Jak3-independent phosphorylation of the critical Tyr 641 residue required for dimerization. This STAT6 version, designated 'STAT6VT' was inserted into the Rosa26 locus preceded by a loxP-flanked Stop cassette. The mice were crossed to CD19Cre mice to enable restricted expression of STAT6VT in B cells.

5.4.2 Confirmation of constitutively active STAT6 in B cells

5.4.2.1 Immunoblotting to detect activated STAT6 in STAT6VT mice

Phosphorylation of STAT6 in CD19Cre_STAT6VT mice should occur at steady state without stimulating the cells with LPS or IL-4. To demonstrate this, splenic B cells where isolated from CD19Cre_STAT6VT mice, WT (Ly5.1) mice, and STAT6$^{-/-}$ mice as a control. These B cells were stimulated with LPS only, or IL-4 + LPS at different time point (0 hrs, 0.15 hrs, 0.30 hrs, 2 hrs and 4 hrs). For detection of phosphorylated STAT6, we used anti- pTyr641 STAT6 antibodies and anti-β-actin antibodies were used as a loading control. At 0 hrs time point, stimulated with LPS only in CD19Cre_STAT6VT B cells we could see STAT6 phosphorylation whereas in WT and STAT6$^{-/-}$ samples phosphorylation of STAT6 was absent. Even at different time points 0.15 hrs, 0.30 hrs, 2 hrs and 4 hrs of stimulation STAT6 phosphorylation was absent in WT and STAT6$^{-/-}$ mice, but in CD19Cre_STAT6VT mice we could see that STAT6 phosphorylation was active at all the different time points (Figure 25). We also looked for total STAT6, which was absent in STAT6-/- but present in comparable levels WT and CD19Cre_STAT6VT samples. Therefore, we can conclude that STAT6 is indeed constitutively phosphorylated in B cells of CD19Cre_STAT6VT mice.

As a next step, we also stimulated B cells with IL-4+LPS for all the three different sample types (CD19Cre_STAT6VT, WT and STAT6-/-). Stimulated B cells were collected at different time points (0.15 hrs, 0.30 hrs, 2 hrs and 4 hrs) and we assessed for phosphorylation of STAT6, and total STAT6. As a loading control we used β-actin. We observed that STAT6 phosphorylation was active in WT and CD19Cre_STAT6VT at different time points (0.15 hrs, 0.30 hrs, 2 hrs and 4 hrs) Figure19B. In addition, the STAT6 activity was only present in cells from WT and CD19Cre_STAT6VT mice whereas in STAT6$^{-/-}$ mice the phosphorylation of STAT6 and a total STAT6 signal was absent at all the time points.

From this experimental set up, we were able to conclude that STAT6 is constitutively active in B cells of CD19Cre_STAT6VT mice and the activated state persists at least for several hours.

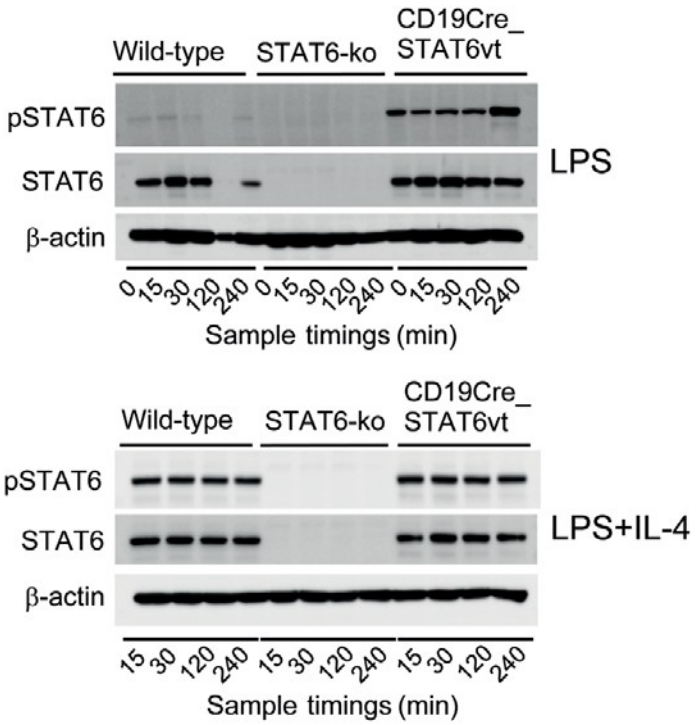


Figure 25: Immunoblotting of activated STAT6 in B-cells of STAT6VT mice. Western blot analysis for detection of phosphorylated STAT6 (pSTAT6), total STAT6, and β-actin in splenic B cells pooled from four individual mice of the indicated mouse strains, stimulated for up to 4 h with LPS or LPS+IL-4. This figure is part of the publication Haase et al. Eur. J. Immunol. (2020) 50:558-567 and used here with permission from the journal.

5.4.2.2 Intracellular staining for flow cytometry

Alternatively, we employed intracellular staining for detecting phosphoSTAT6 in B cells. Splenic B cells where isolated from WT and CD19Cre_STAT6VT mice. These B cells where stimulated with IL-4 for 30 minutes. As a control we used resting B cells from WT and CD19Cre_STAT6VT mice. Resting B cells and stimulated B cells were stained with anti-phosphoSTAT6 intracellularly as per the protocol outlined in the Methods section. Intracellularly stained B cells from both groups of mice were analyzed using flow cytometry.

As a result, after stimulation with IL-4 we could see a greater shift in the phosphoSTAT6 signal in B cells from CD19Cre_STAT6VT mice as compared to B cells from WT mice.

In resting B cells, we could see a slight shift of the pSTAT6 signal in B cells from CD19Cre_STAT6VT mice as compared with B cells from WT mice (Figure 26). Therefore, this experiment demonstrates that STAT6 is constitutively phosphorylated in resting B cells but phosphorylation can be further stimulated by exogenous IL-4 in B cells from CD19Cre_STAT6VT mice.

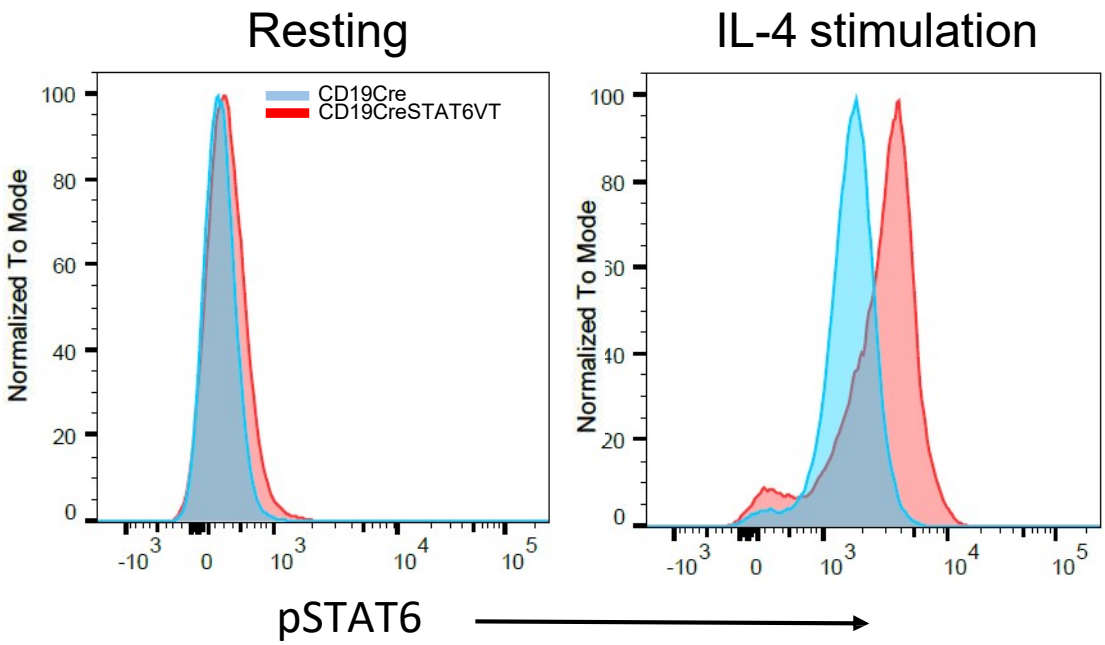


Figure 26: Intracellular staining. Intracellular staining analysis for detection of phosphorylated STAT6 (pSTAT6) in splenic B cells from CD19Cre (blue) and CD19Cre_STAT6VT (red) mice of unstimulated or IL-4 stimulated cells.

5.4.2.3 Immunofluorescence staining

Next, the localization of STAT6 in single B cells from WT or CD19Cre_STAT6VT was determined by immunohistochemistry. Splenic B cells from WT and CD19Cre_STAT6VT mice were first fixed to a glass slide. The slide was subjected to cytospin centrifugation and then stained with anti-STAT6 antibody and DAPI (Figure 27). As a result, we could show that STAT6 was mainly localized in the cytoplasms of B cells from WT mice whereas in B cells from CD19Cre_STAT6VT mice STAT6 was also found in the nucleus (Figure 27).

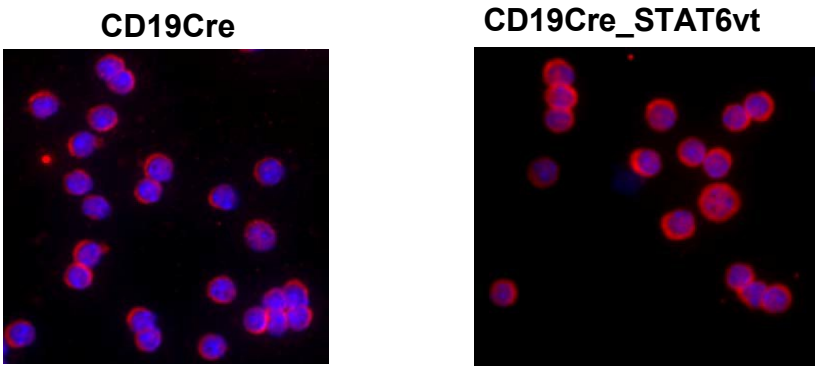


Figure 27: Localization of STAT6 within B cells. Distribution of STAT6 within ex vivo isolated B cells of the indicated mouse strains determined by immunohistological analysis using anti-STAT6 (red) and DAPI (blue). The result from one of two independent experiments is shown.

5.4.3 Development and gene expression analysis at steady state

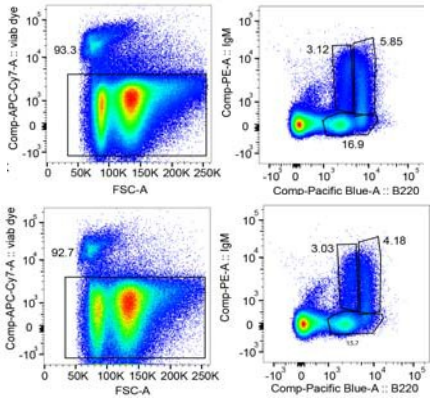
Next, the frequency of mature B cells, immature B cells, follicular B cells, marginal zone B cells and B1 cells was analyzed in bone marrow, spleen and peritoneal cavity from CD19Cre and CD19Cre_STAT6VT mice. Bone marrow cells from CD19Cre and CD19Cre_STAT6VT were stained against IgM and B220 to detect mature and immature cells, respectively. We observed no difference between CD19 and CD19Cre_STAT6VT mice (Figure 28A).

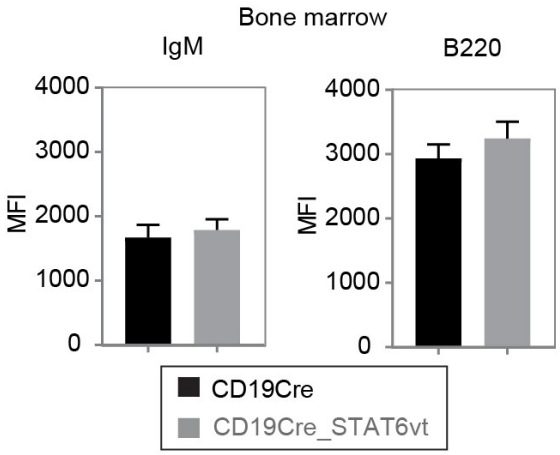
We further analyzed the frequency of follicular B cells and marginal zone B cells by staining with CD23, IgM (follicular B cells) and CD21, IgD (marginal zone B cells). We observed a higher percentage of marginal zone B cells in CD19Cre compared to CD19Cre_STAT6VT mice (Figure 28B). Strikingly we also observed that the mean fluorescent intensity of CD23 is higher in B cells of CD19Cre_STAT6VT mice as compared to CD19Cre controls (Figure 28B). In the peritoneal cavity we observed no difference in B1 cell subsets of CD19Cre and CD19Cre_STAT6VT mice by staining for IgM, CD11b, and CD5 (Figure 28C).

A

CD19Cre

CD19Cre - STAT6VT



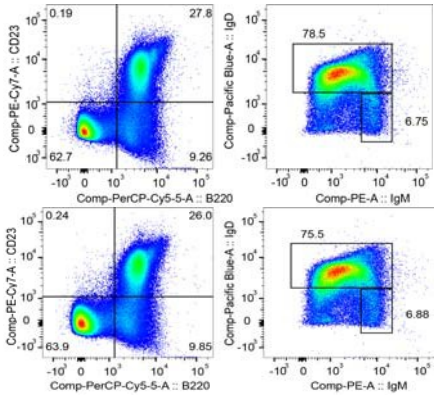


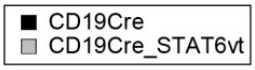
B

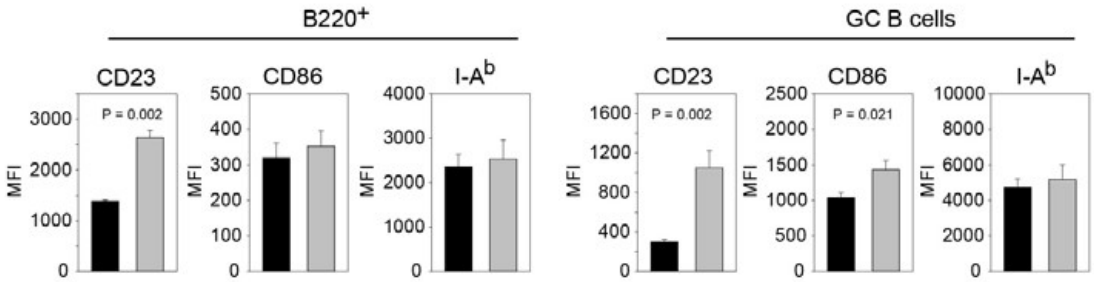
Spleen

CD19Cr

CD19Cre_STAT6 VT







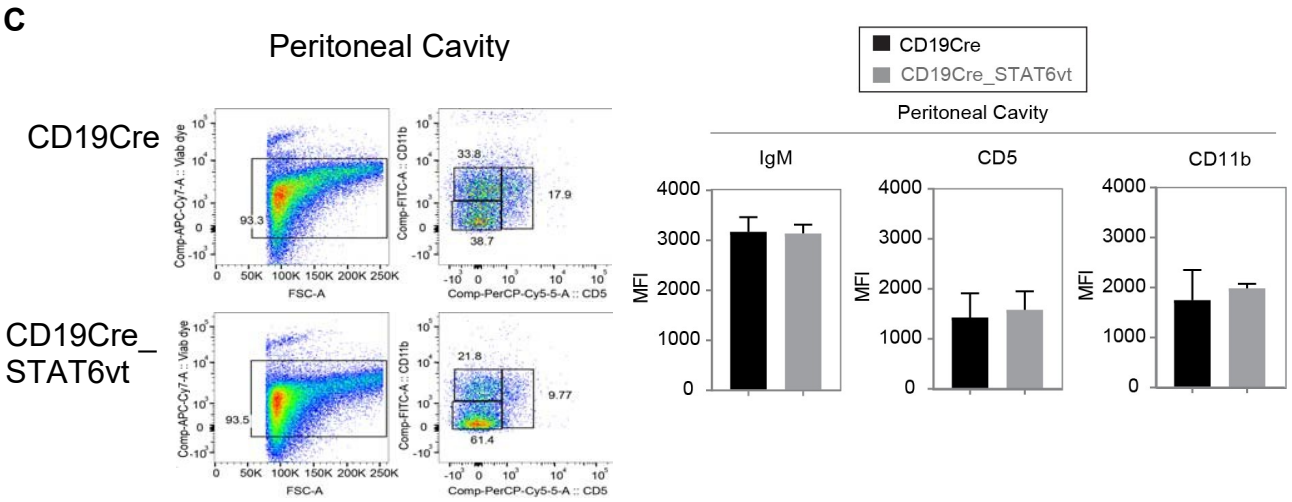


Figure 28: Analysis of B cell subsets. A-C) Flow cytometric analysis of B cells in bone marrow, spleen and peritoneal cavity (PEC). Dot plots show the frequency of pre/pro B cells ($B220^{lo}IgM^{neg}$), immature B cells ($B220^{hi}IgM^{int}$) and mature B cells ($B220^{lo}IgM^{hi}$) in the bone marrow, total B cells ($B220^{+}$) and marginal zone B cells ($IgD^{lo}IgM^{hi}$) within the $B220^{+}$ population in the spleen, and B1 cells in the peritoneal cavity representative for three mice per group. **B)** Bar graphs show mean fluorescence intensity (MFI) of three STAT6 regulated surface markers on all $B220^{+}$ cells and on GC B cells, from naïve mice. **C)** Bar graphs show mean fluorescence intensity (MFI) of IgM, CD11b and CD5 in cells from the peritoneal cavity. Data represent means ± SD of 3-4 independent experiments.

6. DISCUSSION

6.1 Identification of novel STAT6-regulated proteins in mouse B cells by comparative transcriptome and proteome analysis.

The cytokines IL-4 and IL-13 contribute to protective immunity against gastrointestinal helminths but also cause pathological side effects seen in atopic diseases. The cascade of signaling events that follow IL-4/IL-13 receptor binding is mainly transmitted by the signal transducer and activator of transcription 6 (STAT6). The transcription factor STAT6 plays a critical role in B cells during type 2 immune responses by mediating germinal center formation (118) and CSR to IgE and IgG1 (104,105,119,120). To identify STAT6-dependent proteins that may be required for these functions, we performed comparative transcriptome and proteome analysis.

We identified 290 genes that were regulated >3-fold at the transcriptional level by IL-4 in a STAT6-dependent manner. This number is approximately four times higher than previously described by Schroder AJ et al (121) employing a similar in vitro culture system of primary mouse B cells. In this study, they found 70 genes regulated in an IL4-STAT6 dependent fashion. Of the genes they highlighted, we found Caspase6, Bcl-6, Hipk2, Il4ra (upregulated) and Apoe (downregulated) expression being shared with our dataset. In particular, of the Hipk (Hipk1, 2 and 3) family members only Hipk2 was selectively differentially regulated in both datasets. We did not find regulation of CD23 or Krox20 as reported by the previous study. Importantly, our study uncovered regulation of several more genes that were not previously reported. The difference could be explained by the fact that the previous study used another microarray method. Further they analyzed the samples 24 hours after stimulation, whereas our cultures were analyzed on day 4. Moreover, we included anti-CD40 Abs in the culture medium to mimic the signal from Th cells; this stimulus was missing in the previous study (121).

We found that the expression of >400mRNAs was up- or downregulated >3-fold by IL-4 in B cells from WT mice. Most of them were STAT6-dependent, because only 17 mRNAs were regulated by IL-4 in B cells from $STAT6^{-/-}$ mice. This observation shows that STAT6 is the major signaling component for the IL-4-regulated transcriptome in B cells, although the cytoplasmic tail of the IL-4R α-chain contains many docking sites for other signaling molecules, including IRS-1/2, which can further activate the PI3K/Akt and MAPK pathways (75). By label-free quantitative MS analysis, we could identify ~5800 proteins. The expression of essentially all IL-4-regulated and STAT6-dependent proteins was also found to be regulated at the transcriptional level,

excluding a substantial contribution of posttranscriptional mechanism shaping the proteome. A previous proteomic study of the STAT6-regulated proteome used metabolic labeling and 2-D electrophoresis to detect difference in the proteomes of the IL-4 stimulated spleen mononuclear cells of STAT6-/- and wild type mice and MS/MS for protein identification. With this methodology, they identified 49 unique proteins from 21 protein spots to be differentially expressed (122). Of 21 proteins the cellular origin was not defined presumably due to the nature of the experiment in which they used unfractionated spleen mononuclear cells. Our list of 75 up- and 74 down-regulated proteins contains many more interesting candidates that may regulate the function of IL-4-stimulated B cells. There was no overlap between the two studies. This may be explained by the fact that the other study employed a less sensitive experimental approach unlike our highly sensitive mass spectrometry-based proteome analysis.

We selected several up- and downregulated proteins to confirm the quantitative MS data by flow cytometry and western blot analysis. Among the selected proteins was CD79b (also known as Igβ). In a recent study it was found that primary B cells express little Igα and Igβ protein despite a substantial level of the encoding mRNA. IL-4 markedly upregulates Igα and Igβ protein expression and requires STAT6 for this process. Elevated Igα and Igβ proteins form heterodimers that associate with IgM and significantly promote IgM maturation and surface IgM expression, resulting in amplified BCR-initiated signaling that is Lyn dependent (123). CD30 is a member of the TNFR superfamily that is not expressed on resting lymphocytes but was shown to be upregulated by IL-4 in T cells. CD30 was expressed on Th1 cells. Although expression is augmented by IL-4, only CD30 expression is dependent on signal transducer and activator of transcription (STAT)- 6-mediated signaling (124). In the current study, we discovered that CD30 is also induced by IL-4 in B cells; it remains to be analyzed whether this receptor modulates B cell functions during type-2 immune response.

The STAT6 –dependent upregulation of IL-5Rα confirms previous data (125). Interleukin-5 plays a pivotal role in the regulation of Ig secretion from murine B cells. Signaling from IL-5R is mediated, in part, by STAT5, which we identified as another IL-4 induced protein in B cells. Two independent studies further showed that IL-5 promotes the differentiation of IL-4-stimulated B cells to Ab-secreting cells in vitro (126, 127). Therefore, upregulation of IL-5Rα and STAT5 by STAT6 signaling may explain why IL-4-induced and IgE-switched germinal center B cells rapidly differentiate into plasma cells (128).

The most strongly IL-4-induced and STAT6-dependent protein was Thy-1, a phosphatidylinositol-linked membrane glycoprotein that is constitutively and highly expressed in T cells, innate lymphoid cells, neurons, and some other cell types, including basophils and pluripotent hematopoietic stem cells (129, 130). The biological function of Thy-1 is poorly understood, but it may likely be involved in regulating signal transduction from the plasma membrane, because it was shown that thymocytes of Thy-1-deficient mice exhibit augmented TCR signaling and impaired T cell differentiation (135). Ligation of Thy-1 initiates transmembrane signaling pathways that lead to diverse physiological outcomes in different cells (131, 132, 133, 134).

Thy-1 is highly expressed on the surface of CD4+CD8+ double-positive immature thymocytes and downregulated in mature T cells (135). Other studies have shown that LPS+IL-4 stimulated B cells, but not LPS- stimulated B cells, upregulated Thy-1 (136, 137). We confirm and extend these findings by showing that STAT6 expression in B cells is required for this effect. The biological role of Thy-1 on IL-4 stimulated B cells remains to be analyzed.

Unexpectedly, we observed that the signaling adaptor protein SLP-76 was also induced by IL-4 in a STAT6-dependent manner. SLP-76 is required for T-cell and platelet development but has no apparent role in B cell development (138, 139). Rather, normal B cell development depends on the expression of a similar adaptor protein named SLP-65 or BLNK (140). However, it was shown that SLP-76 is expressed in mouse B cells where it binds to the inhibitory receptor CD22 (141, 142). The consequences of IL-4 induced upregulation of SLP-76 in B cells for survival or CSR remain to be analyzed. Several target proteins, probably including SLP-76, are phosphorylated by the tyrosine kinase Syk, which we found to be downregulated by IL-4 in a STAT6-dependent manner in this study. IL-4 is generally regarded as a cytokine that enhances BCR signaling and B cell proliferation (143). Therefore, it would be interesting to investigate whether downregulation of Syk is required for this effect.

Our study further revealed that IL-4 induced the upregulation of DEC205 in B cells. This receptor is highly expressed on dendritic cells where it is used for phagocytosis (144, 145). DEC205 can be targeted for tolerance induction of CD8 T cells via cross-presentation (146). Whether DEC205-expressing B cells have a similar function remains to be analyzed. The surface receptor FcRL1 (CD307a) was described to be primarily expressed on B cells (147). FcRL1 is a type I transmembrane glycoprotein with three extracellular Ig domains and two ITAM

motifs in the cytoplasmic tail (147). This indicates that it could serve as an activating receptor. FcRL1 was found to be upregulated on B cells from patients with acute hepatitis B virus infection (148). However, its biological function has not been characterized so far.

Taken together, we performed comparative transcriptome and proteome analyses to identify novel IL-4-regulated and STAT6-dependent proteins in mouse B cells. This provides the framework to further investigate the biological functions of these proteins during germinal center formation, CSR, and differentiation into memory B cells and plasma cells in the context of type 2 immune responses. A better understanding of these processes could help to develop new therapeutic options for treatment of allergic diseases and helminth infection.

6.2 The role of STAT6 for B cell entry into and localization within the GC.

Our group has previously shown that in mixed bone marrow chimeras the GC formation was dependent on STAT6 expression in B cells (149). IL-4 and IL-13 promote the class-switch recombination to IgG1 and IgE while their role for germinal center (GC) formation is poorly understood. Turqueti-Neves et al. found a dramatic reduction in the numbers of GC B cells when investigating different type 2 immune responses in IL-4/IL-13- deficient mice (149). They further showed that IL-4/IL-13 acts directly on B cells for the formation of a robust GC response. Surprisingly, the frequency of apoptotic GC B cells was not altered in the absence of IL-4/IL-13 and proliferation was even enhanced. The mechanisms that regulate STAT6-dependent GC formation are still unclear and could involve T-B communication or migration behavior of B cells.

The biological consequences of cell exposure to IL-4 or IL-13 are complex since these cytokines can bind to different receptors on immune cells. On one hand, the type I IL-4R composed of the IL-4Rα and common gamma chain (γc) is bound only by IL-4, on the other hand IL-4 and IL-13 share the type II IL-4receptor, which is composed of the IL-4Rα and IL-13Rα1 chains, and thus both receptors may transmit intracellular signals that functionally overlap (150). In type 2 immune responses, TFH cells were reported to derive from TH2 (T helper) cells and express higher levels of IL-4 as compared to Th2 cells (151, 152, 153).

STAT6 phosphorylation occurs in all B cells of the draining LN during type 2 immune responses, not only in B cells that interact with TFH cells in GCs (154). It has further been shown that IL-4 is expressed early after Nb infection at the T-B border (155). IL-4-induced STAT6 activation in B cells was shown to enhance survival of cultured splenocytes by induction of the anti-apoptotic protein Bcl-XL (156) and by regulation of glucose metabolism (157).

With this knowledge, we asked what is the contribution of WT/STAT6 B cells to germinal-center response would be. As shown in the results, our experiment revealed many interesting findings, we found that B-cell transfer experiments could confirm the requirement of STAT6 for entry of B cells into existing GCs. These experiments also demonstrated that antigen-specific activation of B cells has to occur to allow participation in the GC response.

We found that the transferred naïve B cells from SwHEL mice did not acquire a GC phenotype. Surprisingly we observed that the few transferred B cells can successfully become part of a germinal center where endogenous B cells are far more abundant, had already 8 days time to start affinity maturation, and should therefore outcompete the few antigen-specific B cells that are present in the transferred B cell population. Therefore, we draw the conclusion that only antigen specific B cells can enter and migrate into GCs where they compete with endogenous B cells for selection even at late time point (8-12 days after SRBC immunization). The affinity maturation and differentiation into memory B cells or PCs of these “late comer GC B cells” remains to be explored.

6.3 Modulation of B cell responses by constitutively active STAT6

To determine the ability of a constitutively active STAT6 to mimic IL-4-stimulated functions and alter in vivo immune function, a former study generated transgenic mice expressing STAT6VT only in lymphoid tissues using the human CD2 promoter to restrict expression to lymphocytes. In this report, it was found that STAT6VT is sufficient to alter lymphocyte homeostasis, increasing peripheral B cell populations and decreasing T cell populations. Expression of STAT6VT in B lymphocytes led to increased expression of IL-4-induced genes and increased production of IgG1 and IgE, but did not result in B cell activation. In T lymphocytes, STAT6VT expression skewed T cell differentiation toward the Th2 phenotype, and strikingly, induced the activation of T cells, characterized by cell surface marker expression and increased proliferative response. Thus, a constitutively active STAT6 has distinct effects on B and T cell populations (160).

Having established that B cell-intrinsic STAT6 is important for GC formation we further investigated in the current study how constitutively active STAT6 may modulate B cell homeostasis, GC and PC formation. This was performed by characterization of CD19CreSTAT6vt mice which express a constitutively active form of STAT6 (STAT6vt) only in B cells, leaving T cells unaffected.

We found an upregulation of CD23 on the surface of splenic B cells from CD19Cre_STAT6vt mice, but B cell development in the bone marrow appeared to be normal. This could have been expected based on the fact that Cre recombinase is expressed rather late during B cell development in CD19Cre mice (161). Since B cell-intrinsic STAT6 appears to be important for GC formation during type 2 immune responses (118), we expected to see more GC B cells in CD19Cre_STAT6vt as compared to control mice. However, this was not the case. Thus, activated STAT6 alone is not sufficient to drive the GC B cell differentiation program. Further, despite we observed differences in gene expression profile of STAT6 activated cells and several reports identified activating STAT6 mutations in B cell lymphomas (165-167). We neither found obvious alterations in the composition of the major peripheral B cell populations in the spleen or peritoneal cavity nor did we observe mice that developed lymphomas. Furthermore B cell development in the BM also appeared to be normal, which fits to the observation that Cre recombinase is expressed rather late during B cell development in CD19Cre mice (161).

In contrast to CD2:STAT6VT mice where both T and B cells express STAT6VT we did not observe upregulation of MHC-II, and slightly higher levels of CD86 were only detected in GC B cells. This indicates that additional signals that may be derived from activated T cells are required for MHC-II and CD86 upregulation in B cells.

Studies of other STAT proteins have demonstrated a role for serine phosphorylation in addition to tyrosine phosphorylation in the regulation of STAT-mediated gene transcription (162). It is known that STAT6 is rapidly activated in response to IL-4; however, the fate of activated STAT6 is less clear. It was found that during continuous exposure to IL-4, STAT6 activity was sustained for 72 h and that the maintenance of a constant level of activated STAT6 did not require new protein synthesis (163).

In addition, we found that STAT6 in B cells from CD19Cre_STAT6VT mice stimulated only with LPS was phosphorylated in the absence of IL-4 for at least 4 hours. We could also see that STAT6 was mainly localized in the cytoplasms of B cells from WT mice whereas in B cells from CD19Cre_STAT6VT mice, STAT6 was also found in the nucleus. This finding clearly indicated that STAT6 is constitutively activated in each and every cell of CD19Cre_STAT6VT B cells.

7. APPENDIX

7.1 References

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7.4 Declaration

Here with I declare in accordance with the conferral of the degree of doctor from the Faculty of Science of the Friedrich-Alexander University Erlangen-Nuremberg (FAU), Erlangen.

The submitted thesis titled:

“ STAT6-regulated B cell responses “

was written only with the assistance of literature cited in the text.

This is a revised version of a thesis previously submitted to the Friedrich-Alexander University Erlangen-Nuremberg (FAU), Erlangen but not to any other University.

X

Lavanya Mokada Gopal

Heidelberg, 06st of February 2022