Diploma Thesis

Determination of reference intervals for extended lymphocyte phenotyping, lymphocyte stimulation and cytokine levels

Submitted by

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Graz, March 5, 2018

Affidavit

I hereby declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material that have been quoted either literally or by content from the used sources.

Graz, March 5, 2018 *Jonathan Martin Dressen eh*

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Zusammenfassung

Hintergrund: Für ein tiefergehendes Verständnis der Funktionen des menschlichen Immunsystems ist es notwendig sich ein fundiertes Bild von seiner Zusammensetzung in einer gesunden Population zu machen. Ziel dieser Studie war es, die einzelnen Untergruppen der Lymphozyten zu untersuchen und für diese Referenzbereiche zu etablieren, welche die diagnostischen Möglichkeiten der Abteilung für Rheumatologie und Immunologie erweitern sollen.

Methoden: 128 Personen, 56 Frauen und 72 Männer, nahmen an unserer Studie teil. Wir führten eine erweiterte Lymphozytentypisierung sowie eine Lymphozytenstimulation durch, zudem bestimmten wir die Messwerte ausgewählter Zytokine im Blut unserer Probandinnen und Probanden. Wir überprüften unseren Datensatz mathematisch auf mögliche Ausreißer und entfernten diese, falls indiziert. Danach berechneten wir Referenzbereiche. Außerdem überprüften wir den Datensatz der Lymphozyten-Untergruppen auf Korrelationen mit dem Alter der Probandinnen und Probanden, den CRP-Werten und dem BMI. Zusätzlich untersuchten wir unseren Datensatz auf Geschlechtsunterschiede und auf Differenzen im Nikotinkonsum.

Ergebnisse: Wir berechneten Referenzbereiche für die Lymphozyten-Untergruppen, sowohl im prozentuellen als auch im absoluten Bereich, und beschreiben somit die Zusammensetzung des Immunsystems in einer gesunden österreichischen Population. Wir fanden unter anderem eine negative Korrelation zwischen CRP und aktivierten ($CD38^+$) T-Zellen, positive Korrelationen zwischen dem BMI und CRP und negative Korrelation zwischen Lymphozyten und dem Alter. In den meisten Fällen wiesen Frauen sowie Raucher und Raucherinnen eine höhere Zellzahl auf als Männer und Nicht-Raucherinnen und Nicht-Raucher.

Diskussion: Vergleiche mit Studien aus dem europäischen Raum zeigten eine bedingte Übertragbarkeit, was auf unterschiedliche Berechnungen der Referenzintervalle zurückgeführt werden kann. In unserer Studie beobachteten wir zudem zwei Phänomene, welche in ähnlichen Studien ebenfalls beschrieben werden. Zum einen bestehen Veränderungen der Zusammensetzung und Funktionen des Immunsystems im Alter. Zum anderen zeigt sich ein Geschlechtsdimorphismus.

Um die diagnostische Aussagekraft der erweiterten Lymphozytentypisierung auszubauen, ist es notwendig Studien mit ähnlichem Design in Populationen durchzuführen, welche an bakteriellen oder viralen Infektionen erkrankt sind oder an Autoimmunerkrankungen leiden.

Schlagwörter: Lymphozytentypisierung, Lymphozytenstimulation, Zytokine, Referenzwerte

Abstract

Introduction: To further our understanding of the immune system's function, it is necessary to conduct research within a healthy population. The aim of this study was to describe what constitutes a healthy immune system by determining reference intervals for lymphocyte subsets that will also improve the Department for Rheumatology and Immunology's diagnostic methods and are part of the department's certification process.

Methods: 128 persons, 56 females and 72 males, participated in our study after giving written consent. We performed extended lymphocyte immunophenotyping, lymphocyte stimulation and cytokine measurement. After testing for outliers, we determined the reference intervals with a parametric or non-parametric method. We also tested for correlations between the subsets and age, CRP, BMI and cytokines and for differences regarding gender and smoking status.

Results: We successfully determined relative and absolute reference intervals for lymphocyte subsets in a healthy Austrian population. Interestingly, we observed a negative correlation between CRP and activated $CD38^+$ T cells. We observed a positive correlation between CRP and BMI and negative correlations between lymphocytes and age. In agreement with previous reports, we observed three phenomena: First, phenotypical and functional changes the immune system undergoes in the process of aging. Second, a sexual dimorphism in the immune system with a higher percentage and absolute lymphocyte counts in females than in males. Third, in most of the investigated cases, smokers had a distinctive higher cell count than and non-smokers.

Discussion: The comparison of our reference intervals with similar studies conducted in Europe showed only a limited transferability, which is most likely caused by different methods applied to determine the reference ranges.

To improve the diagnostic validity of extended immunophenotyping, further studies, conducted within populations with bacterial infections or autoimmune disorders, are necessary.

Key words: Immunophenotyping, Lymphocyte stimulation, Cytokines, Reference intervals.

Table of contents

List of abbreviations

AHR	Aryl hydrocarbon receptor
APC	Antigen-presenting cell
ASC	Antibody secreting cell
BAFF	B-cell activating factor
BALT	Bronchus associated lymphatic tissue
Batf	Basic leucine zipper transcription factor
Breg	Regulatory B lymphocyte
BMI	Body Mass Index
CAD	Caspase-activated deoxyribonucleicase
Caspase	Cysteine-aspartic protease
CCRx	C-C chemokine receptor type x (x = number)
CD	Cluster of differentiation
CI	Confidence interval
CLP	Common lymphoid progenitor
ConA	Concanavalin A
CRP	C-reactive protein
CSF	Colony-stimulating factor
CSR	Class switch recombination
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXCR-x	C-X-C chemokine receptor type x (x = number)
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EAE	Experimental autoimmune encephalitis
EBF	Early B cell factor
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELR	Glutamic acid, Leucine, Arginine
FACS	Fluorescence-activated cell sorting
Fas	First apoptosis signal
Fas-L	First apoptosis signal-Ligand
FBS	Fetal bovine serum
FDC	Follicular dendritic cell
FoxP3	Forkhead-box-protein 3
FSC	Forward scatter
GALT	Gut associated lymphatic tissue
GATA-x	GATA-binding protein x (x = number)
GC	Germinal center
Gfi-1	Growth factor independent 1

GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hb	Haemoglobin
HLA	Human Leukocyte Antigen
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
IFCC	International Federation of Clinical Chemistry
IFN-x	Interferon-x (x = Greek letter)
IgX	Immunoglobulin X (X = either A, D, E, G or M)
IL-x	Interleukin-x (x = number)
IL1RA	Interleukin 1 receptor antagonist
ILCx	Innate lymphoid cells group x (x = number)
IP-10	Interferon gamma-induced protein 10
IQR	Interquartile range
IRF4	Interferon regulatory factor 4
iTreg	Induced regulatory T cell
kDa	Kilo Dalton
LN	Lymph node
LPS	Lipopolysaccharide
LTα	Lymphotoxin alpha
MHC	Major Histocompatibility Complex
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
MPEC	Memory precursor effector cell
MPP	Multipotent progenitor
MZ	Marginal zone B lymphocytes
NCCLS	National Committee for Clinical Laboratory Standards
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK	Natural killer cell
nTreg	Natural regulatory T cell
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
Pax5	Paired box protein 5
RBC	Red blood cells
RI	Reference interval
RORα	Retinoic acid receptor-related orphan receptor alpha
RORγt	Retinoic acid receptor-related orphan receptor gamma-T
RPMI 1640	Roswell Park Memorial Institute medium 1640
Runx-x_n	Runt-related transcription factor x_n (x_n = number)
RV	Reference value
SCF	Stem-cell factor
SD	Standard deviation
SE	Standard error

SHM	Somatic hypermutation
sIgx	Surface Immunoglobulin x (x = either D or M)
SLEC	Short-lived effector cell
Smadx	Mothers against decapentaplegic homolog x (x = number)
SOP	Standard operating procedure
SSC	Side scatter
STATx	Signal transducer and starter of transcription x(x = number)
T-bet/TBX21	T-box transcription factor
TCF3	Transcription factor 3
TCR	T-Cell-Receptor
TD	T cell dependent
TEMRA	T effector memory CD45RA positive cell
Tfh	Follicular helper T cell
TGFβ	Transforming growth factor beta
TI	T cell independent
TLR	Toll-like receptor
Tmem	Memory T cell
TNFα	Tumor necrosis factor alpha
TNFR-1	Tumor necrosis factor receptor 1
Treg	Regulatory T cell
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{RM}	Tissue resident memory T cell
T1	T cell panel 1
T2	T cell panel 2
VCAM1	Vascular cell adhesion protein 1
VDJ	Variable, Diversity and Joining gene segments (heavy chain)
VJ	Variable and Joining gene segments (light chain)
WBC	White blood cells

List of figures

List of tables

1 Introduction

If we look at the current situation of immunology, it is merely impossible not to think about some of its biggest pioneers: Jenner, Pasteur, Koch and Ehrlich, only to name a few. The first immunological observations are being dated back to the Greek general and historian Thucydides, who witnessed that survivors of the first wave of the plague of Athens showed a reduced susceptibility during further outbreaks (1). It seems safe to say that with the legacies of the aforementioned scientists and the developments of the last 75 years this, branch of medicine has greatly contributed to the health of humanity, by saving lives e.g. through the invention of vaccines, and by gaining a better understanding of the innumerable immunological processes within the human body, thus saving even more lives (2).
This versatile field of biomedical research has an impressive track record but also a serious flaw: considering the recent trend of pharmacies and supermarkets being filled with immune bolstering remedies and foods and taking into account that renowned newspapers publish about the possibilities of defeating cancer with one's very own immune system (3) its actual impact on everyday clinical practice is not (yet) as significant as one might expect (2).
The following study aims to minimize the gap between expectation and reality by taking a step towards the translation of theoretical and experimental research to everyday clinical use.

1.1 The need for conducting research within a healthy human population – the idea behind this thesis

What is missing to make immunology tangible? How is it that immunology is not dominating clinical medicine and biomedical science, especially since current research increasingly shows an involvement of the immune system in the important diseases of modern medicine like atherosclerosis, Alzheimer's disease, cancer and diabetes (2)?
Davis (2008), among others, argues that immunologists need to be able to actually measure the immune system. According to him, as long as we are not able to answer the simple question "How is my immune system?" doctors and scientists will be stuck, unable to fully understand a healthy immune system and diseases with immunological involvement (2,4).

So what can be done to answer this question? Most concepts in immunology are derived from animal model studies, which are still the biggest branch of immunological research and the source for the majority of the immunological discoveries in recent time (4).
The mouse model, more successful than any other animal model, has brought forward many explanations and insights within the last decades, because both immune systems are similar enough to transfer from one to the other (5). Some of the groundbreaking findings that shaped our understanding of the immune system (6) are: the discovery of toll-like receptors (TLR), dendritic cells (DCs), Major Histocompatibility Complex (MHC) respectively Human Leukocyte Antigen (HLA), antigen recognition by T-lymphocytes and the concept of immune tolerance. All of those findings were rewarded with a Nobel Prize within the last 60 years. Undisputable, research based upon mouse models has proved its worth and according to Mestas and Hughes (2004) it will accompany us in our effort to better understand the immune system for years and decades to come (7).

If one looks at the time difference of roughly 65 million years between the emergence of mice and humans and also at the fundamental biological, ecological and behavioral differences, it seems not at all surprising that there are also alterations between humans and mice and their respective immune systems (7).
Some of these differences are of basic anatomical and physiological nature like the distinctive presence of bronchus associated lymphatic tissue (BALT) in mice and the almost lack of it in humans (8). Another example would be the balance of lymphocytes and neutrophils in the bloodstream, which in case of human blood is rich in neutrophils but dominated by lymphocytes in murine blood (9). Some of them are of a quite complex nature like the difference in phenotypes concerning MyD88 deficiency, a major adapter protein for the immune system, which makes mice susceptible to a wide-ranging band of infections whereas humans only show a susceptibility to few pyogenic bacteria (10).
These differences resulted ultimately in the realization that sometimes the transfer from procedures that work in mice to humans fails. Examples for that can be found in various fields like auto-immunotherapy (11), cancer immunotherapy (12) and even in non-immunology medical fields, for example in neurological diseases (13).
The conclusion should not be to abandon mouse research entirely but to realize the necessity not to solely rely on them as models for the immune system, especially since the recent past also showed us quite plainly the limitations of mice in representing the human immune system in all its variety (2,4–7)

Still it proves difficult to give an answer to the question "How is my immune system?", but a solution appears. Before we are able to state which levels of cells are healthy and which cell count might predispose you for any kind of sickness we need to establish what constitutes the healthy immune system and how much biological variability there is (14).

To understand disease we need to understand health first. Research on mice might give important hints and explanations but it is not sufficient to explain the complexity of the human immune system.
Therefore, this study aims to collect basic information about the current state of healthy human individuals, analyzing their lymphocyte subsets, their lymphocyte stimulation capacity and cytokine levels. In the last couple of years the Department of Rheumatology and Immunology at the Medical University of Graz has focused its research on lymphocytes, which has led to a certain expertise when investigating these cell types. The task therefore was to investigate whether or not it is possible to establish reference intervals (RIs) for the lymphocyte subsets and cytokine levels to derive a valid definition of a healthy immune system.
In a second step, these reference ranges are going to be implemented into the University's own clinical Immunology laboratory to provide an expanded diagnostic cell panel when it comes to investigating the cellular composition of rheumatological and immunological patients.

1.2 Lymphocyte subsets

To approach the questions asked in the previous section, it is necessary to take a closer look at the white blood cells (WBC), the main component of the immune system. To receive a detailed insight it is necessary to examine not only the WBC count but also the subjacent levels. Modern methods like the fluorescence-activated cell sorting (FACS), the established method of immunophenotyping (15), (see 2.2 FACS) produces powerful, reliable and repeatable data from blood samples and fragments the inhomogeneous group of lymphocytes into their subsets of B cells and T cells with each of their naïve, effector and memory cell types (16). The discrimination of those subsets is not done morphologically but by using the surface protein markers that are classified in Clusters of Differentiation (CD). When describing a CD as positive ($^{+}$), it means the cell carries that specific protein, negative ($^{-}$)

means that it does not carry it. The procedure of immunophenotyping, the cellular subdivisions with their distinctive cell markers and the antibody staining are explained in chapter two (see 2 Methods).

It is worth mentioning that this study focuses only on some of the vast amounts of cells and subsets within the human immune system. One reason is that when creating this study it was and still is technically impossible to investigate every cell with their subsequent subsets, cytokines and their interdependencies because one will inevitably "not see the forest for the trees".

Another reason is that the some subsets are still subject to active research, on either how to categorize them like the Th9 cells, or what the interdependencies amongst them are. It seems unpredictable whether new cell types and subsets will be discovered. Moreover, the role of any of them might still change

Therefore, this study limits the amount of investigated subsets to a certain number and focuses on cells that have been thoroughly researched in the past, thus representing the current paradigm. The following sections will provide a general idea of the cell groups and subsets investigated in this study, including an overview of their development and functions within a healthy immune system, all summarized in Table 1.

Table 1 Lymphocyte subsets

Subset	Function
Th1	- Production of Interferon gamma (IFN-γ), Interleukin 2 (IL-2), and Lymphotoxin α (LTα) - Eradication of intracellular pathogens by activation of macrophages via IFN-γ - B cell activation and class switching (IgG) - Autoimmunity
Th2	- Production of IL-4, IL-5, IL10, IL-13, IL-25 - Eradication of extracellular parasites - B cell activation and class switching (IgE)
Th17	- Production of IL-17a, IL-17f, IL-21, IL-22 - Eradication of extracellular bacteria and fungi - Autoimmunity

Th1_17	- Production of IFN-γ and IL-17 - Highly proinflammatory potential and association with autoimmune diseases
Regulatory T cell	- Production of IL-10, IL-35 and Transforming Growth Factor β (TGFβ) - Immune tolerance, lymphocyte homoeostasis, regulation of immune responses
Effector memory T cell (T_{EM})	- Protective immunity by migration to inflammation sites and immediate acting as effector cells
Central memory T cell (T_{CM})	- Reactive immunity by homing to secondary lymphatic organs, where they await antigen-recognition-based activation and proliferation to effector cells
$CD8^+$ cytotoxic T cell	- Precise elimination of cells infected with cytosolic pathogens by initiating apoptosis of the target cell via the extrinsic or intrinsic pathway - Production of INF-γ, TNF-α and LT-α and IL-10, initiating both proinflammatory responses and regulating effects
Transitional B-lymphocyte	- Immature B cell, which has already left the bone marrow to complete its maturation process in the spleen.
Marginal zone B-lymphocyte	- Specific B cell subset that circulates the body and populates the spleen's marginal zone, acting as a first line of defense against blood-borne pathogens due to their distinctive location and skill set.
Plasmablast	- Preliminary, rather unspecific version of an antibody secreting cell (ASC), which emerges from the primary focus within the first days of an immune reaction and is relieved by more specific plasma cells soon after.
Switched B-lymphocytes	- Activated and antibody producing B cell that underwent class switch recombination (CSR)
Regulatory B-lymphocyte (Breg)	- Not a distinctive cell line but functional subsets, which are occupied by B, cells at different stages during their development. - Responsible for mediating immune responses.

1.2.1 T-Lymphocytes

The development of all lymphocytes starts, as with every blood cell, with the hematopoietic stem cell (HSC) which proliferates into the multipotent progenitor (MPP) (17). In the next step, the common lymphoid progenitor cell (CLP) marks the beginning of the lymphoid lineage and differentiates to Pre-B-cells, Pre-Natural killer cells (NKs) or immature T-lymphocytes (Pro-T-lymphocytes or Thymocytes) (18). The latter leave the bone marrow via the blood stream to enter the thymus where the maturing process is completed (19). At this stage it is not possible to distinguish between B- and T-lymphocytes morphologically but only with the help of the surface protein marker CD. In case of the immature T cells the leading surface protein is CD3 ($CD3^+$) (18). Upon entering the thymus, the T-lymphocytes move from the cortex to the marrow to complete the maturation. Three major steps are necessary before mature T cells leave the thymus (17). En route they express at first both major CDs for T-Cells, CD4, which binds with Major Histocompatibility Complex II (MHC II) and CD8, which binds with Major Histocompatibility Complex I (MHC I), while later on they downregulate one of them (19).

1. **Test of T-Cell-Receptor:** During this phase the lymphocytes develop a working T-Cell-Receptor (TCR). While contacting thymus epithelial cells the TCR connects with the Major Histocompatibility Complex (MHC) to ensure antigen recognition. By trial and error non-recognizing T cells perish via apoptosis (18).

2. **Test of tolerance towards auto-antigens:** The T cells communicate with professional antigen-presenting cells (APCs) to ensure an adequate self-tolerance towards auto-antigens. T cells that show an heightened activity with auto-antigens perish via apoptosis (18).

3. **Phase of single positivity ($CD4^+$ <u>or</u> $CD8^+$):** Depending on which connection with the MHC was more stable, the T cells now downsize the weaker surface protein. $CD4^+$ T-lymphocytes leave the thymus as mature naïve T-helper lymphocytes, $CD8^+$ T-lymphocytes as mature naïve cytotoxic T-lymphocytes (CTL) and migrate via the bloodstream into secondary lymphatic organs (18).

Upon arrival at the secondary lymphatic organs, the lymphocytes await activation. The appearance of an antigen, presented by APCs, e.g. the dendritic cells (DCs), commences the activation of both the $CD4^+$ and the $CD8^+$ T-lymphocytes. The first step, or first signal, is the connection of the TCR with MHC/antigen-complex with CD4 or CD8 working as co-receptors (19). The second and very essential step, or second signal, is the connection between B7-1 (CD80) and B7-2 (CD86), a co-stimulating molecule on the dendritic cell membrane, and the T cell surface marker CD28 (17,20). As a result, the naïve cells now become effector T cells.

1.2.1.1 $CD4^+$ lymphocytes – T helper cells

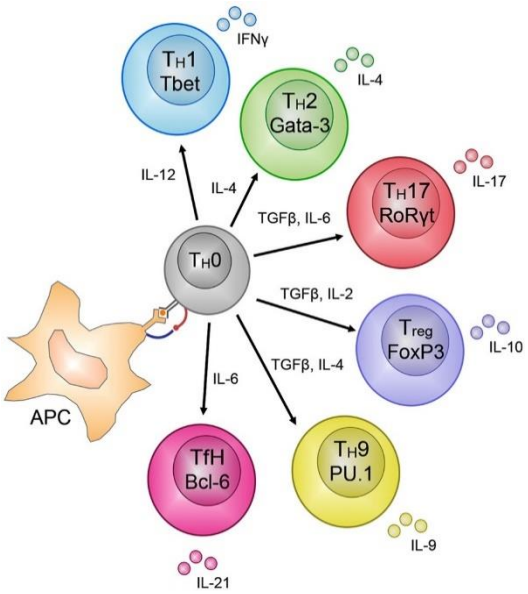


Figure 1 CD4+ subsets
After activation through the APC, the naïve T helper cell (Th0) starts to differentiate. The arrows show the respective activating cytokine. Cell type and master regulator are displayed within the cells, right next to it are the produced key cytokines (21)

T helper cells have in general a supporting role and divide themselves into different subsets with quite diverse functions, which are characterized by the cytokines they produce. Their functions vary from stimulating granulocytes and B-lymphocytes to mediating and regulating the intensity of immune responses (20). Figure 1 (21, with kind permission from Professor S. Turner) provides a quick overview of the $CD4^+$ subsets.

After the aforementioned contact with an antigen via the APCs, the second step of activation and differentiation into the specific subset is determined by the presence or absence of distinctive cytokines, APCs and antigens (22). Helper cells are also able to cross-regulate, thus enhancing or inhibiting the differentiation of their related subsets (18). In the following section a detailed explanation of the differentiation and functions of the major subsets (Th1, Th2, Th17, Th1_17, regulatory T cells) is given.

The IL-9 producing Th9 cells mentioned in Figure 1 are still subject to ongoing research and it remains unclear whether to classify them as an individual subsets of $CD4^+$ T cells or whether they are simply a subset of Th2 cells itself (20).
Follicular Helper T cells (Tfh), predominantly located in secondary lymphatic tissue, play an important role in the B cell mediated humoral immunity (see 1.2.2.4 B cell activation – Plasmablasts, germinal centers and class switching) (20).
Both the Th9 and the Tfh are not the main subject of this study and are purely mentioned to provide a complete overview.

1.2.1.1.1 $CD4^+$-Th1 cells

Differentiation: There are two important cytokines which help naïve helper cells to differentiate into Th1 cells (23). First there is IL-12 which is produced by APCs (24) and second there is IFN-γ which is being secreted by natural killer cells.
The master regulator for Th1 is the T-box transcription factor (TBX21 or T-bet) whose activity depends on both the transcription factors signal transducer and activator of transcription 1 (STAT1), which is activated by IFN-γ and STAT4 which is activated by IL-12 (25,26). The key element for Th1 is the positive feedback loop of both T-bet and STAT4 regarding the IFN-γ production. T-bet is able to increase the production of IFN-γ thus increasing its own activity rate. It also prevents the simultaneous development of Th2 and Th17 by interfering with the IL-4 genes, the Th2 master regulator and one of the Th17 transcription factors' promoter (27,28). Also involved in the promotion of Th1 and the inhibition of the other cell lines are the Runt-related transcription factors (Runx) Runx1 (29) and Runx3 (30).
At a certain point in the process IL-12 and STAT4 upregulate IL-18 receptors that lead to a steady production of IFN-γ, which is independent of inducing triggers, thus guaranteeing constant differentiation of the Th1 subset. T-bet has a self-regulating and presumably protective role by decreasing the amount of IFN-γ in a later stage of the differentiation process (31).

Cytokines and function: Th1 cells are characterized by their production of IFN-γ, LTα and IL-2 (32). IFN-γ has an important role in helping the immune system to eliminate intracellular pathogens by increasing the mononuclear phagocyte system's phagocytic

activity, e.g. in macrophages (18,33). LTα is a protein which is associated with demyelinating autoimmune diseases (34) and IL-2 stimulates the proliferation of T-lymphocytes essentially (35).

Identification: In our study we identified Th1 cells as $CD4^+$, $CD183/CXCR3^+$ and CD196/C-C chemokine receptor type 6 negative ($CCR6^-$) cells.

1.2.1.1.2 $CD4^+$-Th2 cells

Differentiation: The cytokines needed for the differentiation of Th2 cells are primarily IL-2 and IL-4 (20). On a genetic level, STAT6, induced by IL-4, is regarded as the most important transcription factor with GATA-binding protein 3 (GATA-3) as the master regulator (36,37). GATA3 controls the development of the Th2 subset by increasing cytokine production and inhibiting Th1 differentiation by stunting STAT4 (38). Further along in the differentiation process STAT5, which is activated by IL-2 (39), presents itself as an indispensable associate of GATA3 (38,40). IL-6, promoting Th2 and inhibiting Th1 (41), as well as growth factor independent 1 (Gfi-1) (42,43) are completing the group of executors responsible for the Th2 differentiation.

Cytokines and function: The role of Th2 cells is primarily to launch immune responses against extracellular pathogens including helminthes (18). Their cytokine lineup consists of IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and Amphiregulin (32).
Interleukin 4 is a key element of the positive feedback loop (44) in the Th2 differentiation process (45) and also responsible for the IgE class switching of B cells (46). Furthermore, it intensifies the bodies' proinflammatory response by inducing e.g. IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and vascular cell adhesion protein 1 (VCAM1) (47).
Interleukin 5 is the initiating cytokine of eosinophils (48,49). Interleukin 9 has an activating influence on mast cells, B cells, eosinophils and neutrophils (20) whilst increasing the airway epithelia's mucus production (50). Interleukin 10 regulates the immune reaction (51), decreasing the activity of immune cells after pathogens have been cleared by inhibiting Th1 proliferation (52) and dendritic cells (53). Interleukin 13 is the cytokine of choice when it comes to eliminating the aforementioned helminthes (20) but it also increases airway

hypersensitivity (54,55). Interleukin 25 both initiates and amplifies the Th2 proliferation by increasing the production of i.a. IL-4 (56) and also IL-13, which inhibits the Th17 response (20). Amphiregulin, as part of the epidermal growth factor (EGF) family, induces the epithelial cell proliferation (32) but also plays a role in the expulsion of helminthes (57). Especially the functions of IL-4. IL-9 and IL-13 show Th2's involvement in asthma and other allergic diseases (58,59).

Identification: In our study we identified Th2 cells as $CD4^+$, $CD183/CXCR3^-$, $CD194/CCR4^+$ and $CD196/CCR6^-$.

1.2.1.1.3 $CD4^+$-Th17 cells

Differentiation: The Th17 cell line differentiation can be divided into three steps (20).
The first stage is being controlled by TGFβ and Interleukin 6 (60). TGFβ plays a fundamental role in the development of this subset due to the fact that it is involved in every phase of the process (61,62). Interestingly, it also is a key cytokine of Treg development. Only the concentration of the growth factor determines the fate of the naïve helper cell, while a high concentration of TGFβ leads to induced regulatory T cells (iTregs) (63) and a low concentration, combined with the presence of IL-6, promotes the differentiation of Th17 (60–63).
Together with Interleukin 6, TGFβ activates the master regulator retinoic acid receptor-related orphan receptor gamma-T (RORγt) (64). Furthermore, TGFβ's main task in the first phase is to enhance the activation of the transcription factor STAT3 (65), which then induces the expression of RORγt (20).
Another transcription factor involved is Runx1, which in a mutual reaction with RORγt enhances the Th17 differentiation (20). Like TGFβ, Runx1 is not working exclusively in favor of the Th17 cell line because together with FoxP3 and T-Bet it is able to suppress the differentiation process (66). Other contributing transcription factors in this phase are retinoic acid receptor-related orphan receptor alpha (RORα) (64), Aryl hydrocarbon receptor (AHR) (20), basic leucine zipper transcription factor (Batf) (20) and the protein Interferon regulatory factor 4 (IRF4) (20).
In the second phase of the differentiation IL-21 is the leading cytokine, even though it is not Th17's major cytokine (IL-17 is) (20). This is a different process compared to the

development of Th1 and Th2, where their respective major cytokines (IFNγ and IL-4) were responsible for strongly amplifying the subset's differentiation (20). Interleukin 21 together with TGFβ is self-amplifying the subset's expansion, i.a. by activating STAT3 (20,32). Responsible for the third and last phase is Interleukin 23, a product of APCs (32). In this step, the cytokine's main task is to ensure the subset's further growth and survival (60). Il-23 is also able of activating STAT3 (67).

Cytokines and function: Th17 cells are in charge of combating extracellular bacteria and fungi (68). Their major effector cytokines being IL-17a, IL-17f, IL-21 and IL-22 (32). Interleukin 17 is responsible for a proinflammatory reaction by inducing corresponding cytokines like IL-1, IL-6, and tumor necrosis factor α (TNFα). It also helps to generate chemokines like IL-8 to enable chemotaxis, thereby recruiting and activating neutrophils (69). Interleukin 21 activates NKs, other T cells and assists B cells in their differentiation to plasma and memory cells (70). Interleukin 22 acts as a mediator of the immune response and enhances the production of antimicrobial peptides and promotes cell proliferation in order to protect local tissue (71). Multiple studies with animal models show Th17's association with autoimmune diseases as the reason why they are subject to thorough research (32,72).

Identification: In our study we identified Th17 cells as $CD4^+$, $CD183/CXCR3^-$ and $CD196/CCR6^+$.

1.2.1.1.4 $CD4^+$- TH1/17 cells

Differentiation: In the chapters above, Th1 and Th17 cells have been respectively defined as independent subsets of the $CD4^+$ helper cells. Within the last twenty years, a new subset has been discovered. These cells produce both the characteristic cytokines of Th1 cells, IFN-γ, and Th17, IL-17 and have been described as either Th1/17 (73) or Th17/1 (74–77) or IFN-γ producing Th17 cells (74). Essential for the development of this subset is the presence of both IFN-γ and IL-12. The former is responsible for activating the transcription factor T-bet, which induced the expression of an IL-12 receptor. Through this receptor, the cell is susceptible for IL-12 and, when activated, produces T-bet. In the end, the Th1/17 cells co-express both T-bet and RORγt. Since the Th1/17 cells are able to combine the proinflammatory potential of the original subsets, multiple studies attribute the Th1/17 cells

to be responsible for major pathogenic processes within autoimmunological diseases (74,78–80). The depth of the relationship between Th1, Th17 and the Th1/17 cells is still subject to ongoing research (74).

Cytokines and function: The Th1/17 cells are able to provide help to B cells, inducing the production of all antibodies but IgE (74), thus intensifying the body's immune response but show a reduced cytotoxic activity and ability compared to Th1 cells (74). Furthermore Th1/17 cells are less predisposed to be inhibited by Tregs (74).

Identification: In our study we identified Th1/17 cells as $CXCR3^+$ and $CCR6^+$.

1.2.1.1.5 $CD4^+$-Regulatory T cells

Differentiation: In current scientific literature two types of regulatory T cells (Tregs) are being identified (20). The natural regulatory T cells (nTregs) and the induced regulatory T cells (iTregs). This paragraph will only address the iTregs' differentiation process. The defining difference for the two groups is their place of origin. The former develop within the Thymus and leave it with their key transcription factor FoxP3 already expressed. The latter derive from mature naïve $CD4^+$ T cells within secondary lymphatic organs (81). The distinctive contribution of each type of Treg to the human bodies' tolerance towards auto and foreign antigens is unknown (32).

As mentioned above, TGFβ is the major cytokine for both the Th17 and the iTreg differentiation (82,83). In case of the latter the growth factor activates the expression of the most important transcription factor FoxP3 (84,85).

The proteins small body size mothers against decapentaplegic homolog 2 and 3 (Smad2, Smad3) are transcriptional modulators activated by TGFβ (83). They in return induce FoxP3 expression (86) and are also able to inhibit RORγt and therefore the Th17 development (87).

STAT5 is another important transcription factor since it enhances FoxP3 expression on the one hand (88,89) and blocks STAT3's binding to the gene locus on the other hand, thus favoring iTreg differentiation over Th17 differentiation (20). The other relevant transcription factor is the nuclear factor of activated T cells (NFAT) which interacts with FoxP3 (90,91).

Cytokines and function: Both iTregs and nTregs play an important role in the bodies self-tolerance and in the moderation of immune responses to avoid damage due to autoimmunological pathological processes (92,93), making them very interesting to the understanding and treatment of autoimmune diseases and graft rejections after transplantations (32). Their key effector cytokines are IL-10, IL-35 and TGFβ (20). Interleukin 10, supported by interleukin 35, is able to appease the immune system's response after an inflammation eliminated the site of all pathogens (51,94). In doing so IL-10 actively prevents the present immune cells from inflicting too heavy damage to the local tissue (20,32). Their role is supported by research that shows IL-10's involvement in the cure of Inflammatory Bowel Disease (IBD) (95,96) and a mediating influence on Experimental Autoimmune Encephalitis (EAE) (32). As a result of Interleukin 10's and TGFβ's influence, IgE production is being restricted, hinting the two cytokine's importance when it comes to allergic reactions (97).

Identification: In our study we identified Tregs as $CD4^+$, $CD25^+$ and $CD127^-$. Although $CD25^+$ is expressed by all activated T cells, it shows the highest concentration within Tregs, making its measurement a useful tool when separating Tregs from other T cells (98).

1.2.1.2 $CD8^+$ lymphocytes – Cytotoxic T-lymphocytes

Differentiation: $CD8^+$ lymphocytes migrate to the margin of the T cell zone within a lymph node (LN) (99). They encounter antigen-bearing macrophages and DCs which commences the activation of the yet naïve CTLs (100). The majority of the developing cytotoxic lymphocytes are short-lived effector cells (SLECs) that perish rapidly after the infection has been fought off, the minority of memory precursor effector cells (MPECs) are determined to become memory cells later on (101,102). Until now it remains unknown which signal is responsible for the development of either SLECs or MPECs (100).
Within the first seven days of activation a $CD8^+$ T cell goes through up to 19 cell divisions (103) with a maximal division rate between two and six hours (100,104). To facilitate this clonal expansion T cells increase their metabolism drastically and absorb high amounts of glucose, amino acids and iron (105).
One important cytokine for CTL differentiation is Interleukin 12 (106) which, among other tasks, increases the growth of T cells by inducing T-bet (107). Another major cytokine and

differentiation factor is Interleukin 2 by activating variable molecular pathways which promote CTL differentiation, e.g. the increased activity of transcription factor STAT5 or the activation of mitogenic kinases like PI-3K and MAPK (108). Blimp-1, a transcriptional repressor activated by IL-2, plays an important role in the development of the SLECs. Its main function is to inhibit IL-2 production which leads to a self-regulating function within the SLEC population (108). Other relevant transcription factors are Runx3 and Tcf-1 (109).

In the next step the effector CTLs need to migrate to the site of infection to engage the pathogens. In order to do so they start to express CXCR3 (110). When chemokines bind to the receptor the lymphocytes are able to express integrines to enable their chemotactic migration (110).

Cytokines and function: CTLs play an important role in the body's defense against cytosolic pathogens, particularly viruses. When a virus has infiltrated a cell it is out of reach for humoral immune mechanisms. The infected cell signals its contagion to its surrounding with the help of the MHC I molecules, acting as non-professional antigen-presenting cells and offering the antigens to immune cells like the cytotoxic T cells. (19)
After antigen recognition, they use three major functions to combat intracellular pathogenic agents, two of them leading to the target cell's death via apoptosis. The common denominator of those two functions is the activation of cysteine-aspartic proteases (Caspases), which can be subcategorized into initiator caspases, which activate the effector caspases (18). They in return cleave the inhibitory elements of the caspase-activated deoxyribonuclease (CAD) which degrades the deoxyribonucleic acid (DNA) (18). The activation of the initiator caspases leads to a cascade that amplifies their effect.
The first function, the extrinsic pathway of apoptosis, initiates the programmed cell death via the cell surface. The CTLs transmembrane protein first apoptosis signal-ligand (Fas-L) and the secreted TNF-α communicate with the respective receptors Fas/CD95 and tumor necrosis factor receptor 1 (TNFR-1) expressed on the target cells (17). After Fas-L binds with Fas, a signaling pathway activates initiator caspases 8 and 10, which in return activate the effector caspases 3, 6, and 7, ultimately initiating the cell's apoptosis (18).
The second function, the intrinsic pathway, uses cytotoxic elements within the T cells to activate the apoptosis (111). Secreted in granules, the cytotoxic effector protein Perforin creates pores within the cell membrane of the target cell, facilitating Granzymes and Granulysin to reach the target cell's cytosol where Granzyme B triggers apoptosis by

activating initiator caspase 9 which activates effector caspase 3 and leads to the degradation of DNA (19). The intrinsic pathway also cleaves anti-apoptotic proteins that eventually disrupts the outer membrane of the mitochondria, leading to the release of cytochrome c, which amplifies the apoptosis process (18). These functions enable the CTLs to surgically remove single cells from a collective tissue without damaging healthy cells or starting an inflammatory reaction.

Furthermore, CTLs are able to release IFN-γ, TNF-α and LT-α (111). These cytokines inhibit viral replication, increases the expression of MHC I in infected cells, thus increasing the likelihood that those cells are being targeted by the immune system and also activate macrophages, promoting their chemotaxis to the site of infection (112).

In recent years a new function of CTLs has been discovered (113). Several studies confirmed that cytotoxic T-lymphocytes also produce Interleukin 10 which acts in a regulatory way, inhibiting the immune response to avoid the infliction of too much damage to the inflamed tissue (113–115). Palmer et al. (2010) and Trandem et al. (2011) even found out that at the peak of the immune reaction CTLs produce the most IL-10 (114,115).

Identification: In our study we identified naïve CTLs as $CD8^+$, $CD45RA^+$ and $CD197/CCR7^+$.

1.2.1.3 Memory T-lymphocytes

With the help of surface protein markers like cytokine or chemokine receptors memory cells can be subcategorized into three major groups (116). A lot of the current research is mostly based on $CD8^+$ lymphocytes, whereby some argue that memory cells differentiate and function equally, regardless whether they are $CD4^+$ or $CD8^+$ (117). The following chapter will explain both $CD4^+$ and $CD8^+$ without distinguishing between them except when explicitly stated.

There are central memory cells (T_{CM}) and effector memory cells (T_{EM}). A third group consists of tissue resident memory T cells (T_{RM}). Since the samples in this study were gathered from peripheral blood, T_{RM} are not being investigated in this study.

It should be noted that memory cells are an extremely heterogeneous group of cells. Especially during antigen-driven activation, memory cells express a vast amount of surface

markers transiently, making it difficult to categorize them. The below mentioned distinctions between the particular cell lines therefore apply to resting cells (118).

Central memory cells: T_{CMs} provide reactive memory by traveling to secondary lymphatic organs where they await activation and then differentiate and proliferate to effector cells (18). They are $CCR7^+$, $CD45RA^-$ and $CD45RO^+$ and also express CD62-Ligand which together with CCR7 facilitates the extravasation to the lymphatic system (119). After they encounter an antigen via the TCR, they start to produce primarily IL-2 while later on IFN-γ as well as IL-4. While T_{CMs} consist of Th1, Th2 and CTLs, $CD4^+$ helper cells are predominant.

Effector Memory cells: T_{EMs} provide protective memory, because they migrate to sites of inflammation and are ready to engage pathogens immediately (118). Therefore, they are present in the bloodstream, the lungs, liver and gut. They are $CD45RA^-$ and $CCR7^-$. Compared to T_{CMs} the effector memory cells are able to react immediately to a pathogen encounter by producing large amounts IFN-γ, IL-4 and IL-5. The $CD8^+$ T_{EMs} possess numerous vesicles with perforin and Granzymes. CTLs are also the predominant cell line amongst the T_{EMs}.

The third T memory subset are T effector memory RA-positive cells (TEMRA), which express CD45RA and are $CCR7^-$. Regarding their function, TEMRAs resemble effector cells by quickly reacting against pathogens (119).

Both T_{CMs} and T_{EMs} respond firmly to antigen stimulation but T_{CMs} differentiate heterogeneously. Some of these cells are committed pre-Th1 and pre-Th2 cells, the majority however is uncommitted and shows a certain flexibility regarding the priming effect of influencing cytokines as well as the commitment to the production of a cell line's specific cytokine (118).

Identification: In our study we identified T_{CM} as $CD4^+$ or $CD8^+$, $CD45RA^-$ and $CD197/CCR7^+$. T_{EM} were identified as $CD4^+$ or $CD8^+$, $CD45RA^-$ and $CD197/CCR7^-$. Thirdly, T effector memory RA-positive cells (TEMRA) were defined as $CD197/CCR7^-$ and $CD45RA^+$.

1.2.2 B-lymphocytes

The process of B cell development is quite complex, with some parts still being researched (120). To explain every step thoroughly would go beyond the scope of this thesis. Therefore, the following section will provide a summary of B cell development and B cell subsets relevant for this study.

1.2.2.1 Central development, maturation and self-tolerance

Just like T-lymphocytes, B-lymphocytes have their origin in the HSC. B-lymphocytes mature in the bone marrow (121). Misleadingly, the letter B in B-lymphocytes derives not, as commonly assumed, from the bone marrow but from the Bursa of Fabricius, a lymphatic and hematopoietic organ found in birds (18).
The process of B cell development in mice is has been systematically researched, whereas the process in humans is not yet fully understood (120). What can be derived from murine models is that for the survival of the B cell precursors, the initial communication between the HSC/MPP and the bone marrow's stromal cells is essential (122). The connective tissue cells provide signals for activation of key genes within the differentiation process by cell to cell contact, cytokines and chemokines (e.g. CXCL12) (both soluble and membrane-bound (e.g. stem-cell factor SCF)) (18,123). The transient expression of various transcription factors, e.g. transcription factor 3 (TCF3), early B cell factor (EBF), paired box protein 5 (Pax5) (123) and cell-surface receptors in the course of the different steps of development leads to the first definite B cell stage, the pro-B cell (124).

Antibodies, both soluble and membrane-bound as BCRs, consists of light and heavy chains, each with constant and variable regions (18,125). The pro-B cell is characterized by the rearrangement of the variable (V), diversity (D) and joining (J) gene segments of the heavy-chain genes (126,127). This development step contributes greatly to the B cell diversity. To test the functionality of the gene recombination, a receptor is being formed out of the heavy chains and an imitation that resembles the light chains, a so called surrogate chain, because the light chain genes have not yet rearranged (128,129). This receptor is called the pre-B cell receptor and the successful recognition of an antigen marks the transition from pro-B to pre-B cell (130,131).

Within the pre-B cell, among others, three major processes are taking place to ensure the further development of this subset. The first is the allelic exclusion, which allows only one of the heavy chain and light chain alleles to be expressed, guaranteeing that the later B-lymphocyte is only specific to one antigen (129). The second process is the VJ rearrangement of the light chain gene loci (126). The third is the isotopic exclusion. Similar to allelic exclusion, this step ensure that the lymphocyte expresses only one type of antigen receptor by allowing the previously selected allele to express only one type of the light chains, either κ or λ (132). After the successful rearrangement, membrane-bound Immunoglobulin M (IgM) is expressed as part of the B cell receptor (BCR), which consists of the IgM and a co-receptor (CD19, CD21 and CD81). The cell is now an immature B-lymphocyte.

Before the immature lymphocytes can leave the bone marrow, central tolerance (because it is being generated in a central lymphatic organ) must be tested by exposing the BCR to auto-antigens (133). Only if the cells show no autoreactivity it can enter the bloodstream to migrate to the spleen where it completes its maturation. However, if the receptor shows autoreactivity four different options exist. The first option is possible if the BCR recognizes a multivalent antigen. In this case, further light chain gene rearrangement can take place, which is called receptor editing, leading to a newly configured receptor which shows no autoreactivity (134). The rearrangement can continue until either a non-autoreactive receptor is produced or until no further genes are available (18). If the receptor editing fails the cell enters clonal deletion, the second option, leading to its death by apoptosis (135). The third option is available if the immature B cell binds an auto-antigen with low affinity and no cross-linking. It is going to remain in an unresponsive state called anergy from which they neither can be activated nor do they die (18). The fourth option is called immunological ignorance, describing a state where the autoreactive cell does recognize an auto-antigen but for some reason does not trigger a response, at least at this certain moment (18).

Central tolerance can be summarized as a compromise between a broad repertoire of antigen recognizing receptors and a certain level of autoreactive B cells (133–135). It also describes a process that the immature B cells undergo before they enter the bloodstream.

1.2.2.2 Peripheral maturation and self-tolerance – Transitional B-lymphocytes

B-lymphocytes leave the bone marrow and travel via bloodstream towards the spleen. As they are not yet totally mature they are described as transitional B cells (136). Even though

they already display central tolerance, they still need to develop a peripheral tolerance. Some auto-antigens are compartmentalized and are not present in the bone marrow because they are tissue-specific. Some B-lymphocytes might not encounter their self-antigens until they reach the bloodstream, the spleen or specific organs (18). The process of self-tolerance is similar to the one in the bone marrow and results in the same fate: survival by rearrangement, clonal deletion or anergy (18).

The still immature B cells are characterized by the expression of large amounts of membrane-bound or surface-Immunoglobulin M (sIgM) and low levels of sIgD (18). In order to finalize the maturation process they need to reach the follicles in the spleen (121). Every peripheral B cell circulates through these follicles. Due to a limited number of follicles, mature and immature B cells are forced to compete with each other for entry, resulting in the death of almost half of the immature population (18). This is because mature peripheral B cells are long-lived but immature cells die within days if they are not able to reach a follicle and receive signals that enable their survival. Those signals are provided by follicular dendritic cells (FDCs) within the spleen, who produce, among others, the B cell activating factor (BAFF) (137). Inside the follicles the transitional B cells have to pass the T1 and T2 stage in that order to finish maturation (138). The distinction between those two steps is the absence (T1) or presence (T2) of CD21 (19). In the last step the $CD21^+$ T2 cells upregulate sIgD and are from now on long-lived mature B cells (18).

Identification: In our study we identified transitional B cell as $CD19^+$, $CD38^+$ and IgM^+.

1.2.2.3 B-1 and B-2 cells:

Mature B cells can be subcategorized into three subsets: B1 cells, follicular B cells and marginal zone B cells, the latter often summarized as B2 (18,139).

B1: In the sections above, B cells were always described as an important part of the adaptive immune system, which is characterized by a highly specific response to pathogens (140). It is however not characterized to respond as fast as the unspecific innate immune system does (140,141). However, there is a group of B cells that is being considered to be a part of the innate immune system. Even though they are not the focus of this study and their existence in humans is controversially debated because most of the research is based on murine models (142), they are mentioned and explained to provide a holistic portrayal of B-lymphocytes.

These innate B-lymphocytes are called B1 B cells. They originate from precursor cells within the fetal liver and have their primary location not in the secondary lymphatic organs but in the bodies' peritoneal and pleural cavities (142). They continuously produce natural antibodies (IgM), for which they do not need the presence of a pathogen and can be activated by T cell independent (TI) antigens (143). Therefore, they are able to initiate a rapid but unspecific immune response, long before the adaptive immune system takes over (141).

B2: The majority of the mature B cell population developing in the spleen consists of B2 B cells. The two subgroups are the follicular B cells and the marginal zone B cells (MZ) (121). The first are the follicular B cells, the predominant B2 subset, which can be found in the lymphoid follicles of secondary lymphatic organs but also circulate through the body. They are capable of a TI reaction to antigens but prefer to react in a T cell dependent (TD) manner (143).

The second group are the marginal zone B cells. They populate a specific zone within the spleen where the red pulp merges with the white pulp (18,143). Since this position is a crossover between the immune system and the bloodstream, it exposes them to the large amounts of blood that circulates through the spleen (144). Marginal zone B-lymphocytes occupy a special duty among the B cells because they represent the first line of defense against blood-borne pathogens, especially encapsulated bacteria (145). While murine MZs are sessile to the marginal zone within the spleen (144), it has been discovered that human MZs circulate the body (146), thus enabling their measurement in our study.

Identification: In our study we identified marginal zone B cells as $CD19^+$, $CD27^+$ and IgD^+.

1.2.2.4 B cell activation – Plasmablasts, germinal centers and class switching

There are two ways to activate a mature naïve B-lymphocyte. Just as T-lymphocytes need both the signals through the TCR and a second signal via CD28 and B7.1/B7.2, B-lymphocytes also need a second signal. This signal can be provided either by T cells (TD) or by the antigen itself (TI) (121).

In order to communicate with the T cells, B cells first internalize the antigen via the BCR, degrade it and then present antigen fragments via a MHC II/peptide-complex on their cell surface, where it is recognized by a follicular helper cell's TCR. This happens only, if the T-cell has already been activated by the same antigen, a mechanism called linked recognition

that is ought to prevent unnecessary lymphocyte activation (18). B cells that came in contact with their antigen but do not encounter a correspondent T cell die within 24 hours (18). The Tfh in return provides the B cell with important signals for survival and proliferation (147). Amongst others, Tfh activate the non-canonical pathway of nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) via the B-cell's CD40 and the production of cytokines, e.g. IL-21 (18). Those steps in return enhance the activation of transcription factors and the production of anti-apoptotic proteins, therefore enabling the further differentiation of the B-lymphocyte.
In case of the TI pathway, the second signal is provided by the antigen itself via the B cell's very own TLR, which detects pathogen-associated molecular patterns (PAMPs) like bacteria's lipopolysaccharides (LPS) (18). The signaling through the TLR activates NFκB in the canonical way but just as the TD signal, it leads to the B cell's survival and proliferation (18).

Within secondary lymphatic organs, the lymphocytes are located in two distinct areas. The T cells occupy the T cell area and the B cells the B cell area, also known as the primary lymphoid follicles (18). Both zones are also populated with stromal cells and DCs that provide a sort of scaffolding for the lymphocytes and ensure their survival by producing cytokines and chemokines (18).
In the course of an immune reaction, antigens are being transported to lymph nodes via lymph vessels or to the spleen via blood. In LNs, soluble antigens are detected by follicular B cells. In the spleen, MZ carry the antigens to FDCs, which in return present it to the naïve mature B cells. T-cells receive the antigens via DCs. After activation, a part of the T-cell proliferates to effector cells while another part migrates to the primary lymphoid follicle's margin to communicate with B cells that were activated by the same antigen. Their interaction provides both cells with the respective signal to differentiate and proliferate.

While the T cell's development has already been explained in the previous sections, the B cells remain in a differentiating and proliferating state for few days. In the next step, some of the B cells regroup and form the primary focus, which represents the first stage of the humoral immune response. Within the primary focus, some of the B cells differentiate to plasmablasts, a preliminary, yet not highly specific antibody secreting cells, which continues to proliferate still. After a couple of days, those plasmablasts die and the remaining B cells

of the primary focus differentiate to long-lived plasma cells that travel to the bone marrow while steadily producing and releasing antibodies.
Those B cells that did not enter the primary focus migrate back to the primary lymphoid follicles, accompanied by Thfs. This accumulation of activated B and Tfh cells is no longer called the primary lymphoid follicle but a germinal center (GC) or a secondary lymphoid follicle (148). Within the germinal center the affinity maturation takes place, which consists of two major mechanism that ensure the adaptive immune system's ability to produce highly specific antibodies. Somatic hypermutation (SHM) is a process of mutation with a much higher mutation rate than normal within the V regions of antibodies, which after multiple cycles ultimately leads to highly specific B cell clone. The second mechanism is the following clonal selection and expansion of those B cells that show the highest affinity to the antigen and are therefore able to successfully compete over the Tfh's survival signals (147). The remaining, non-specific B cells are expendable and perish due to neglect via apoptosis. However, the survivors either differentiate to plasma cell or memory cells (148).

Another important mechanism for activated B cell is the Tfh mediated immunoglobulin class switching (147). The first antibodies produced by naïve B cells are IgM and IgD. After the isotype switching the B cells are also able to produce IgG, IgA and IgE. Class switch recombination is a process of nonhomologous gene recombination within the so-called switch regions in the constant region of the heavy chain's DNA. It should be noted that this is not a part of the affinity maturation because the variable region of the antibody genes is not altered. The CSR simply enables a group of daughter B cells to produce a different kind of antibody with the same antigen affinity. The process of CSR is influenced and directed by the cytokines released from the follicular T helper cells. B-lymphocytes that underwent CSR are called switched B cells.

Identification: In our study we identified switched B cells as $CD19^+$, $CD27^+$ and IgD^- and plasmablasts as $CD19^+$, $CD38^+$ and IgM^-.

1.2.2.5 Regulatory B-lymphocytes

Regulatory B cells are a subset of B-lymphocytes with an entirely different occupation. As the name suggests, Bregs initiate regulatory effects that soften the intensity of a pro-

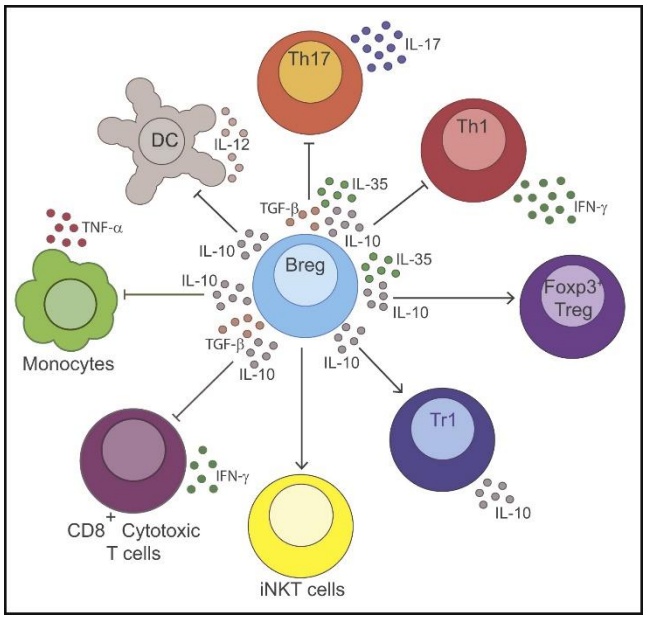


Figure 2 Regulatory B cell
The key effector cytokines IL-10, IL35 and TGFβ show the both inhibiting effects on Th1, DCs, monocytes and CTLs and the enhancing effects on regulatory T cells and NKs (150).

inflammatory response, thus preventing too extensive immune reactions, tissue damage and chronic immunopathology (149).

In that way, Bregs are quite similar to their regulatory siblings among the T-lymphocytes. While Tregs are mainly characterized by their major transcription factor FoxP3, a similar identifying molecule for the B cells has yet to been found (150). Latest studies suggest that there is no distinctive Breg lineage, but instead both mature and immature B cells (150) as well as plasmablasts (151) are all able of regulating effects and the distinctive milieu of inflammation is responsible for Breg differentiation (150). Even though it is difficult to explain the origin and the development of Bregs, the research of their effector functions has been more successful (152). Their cytokine repertoire consists of IL-10, IL-35 and TGFβ and with those, Bregs are able to suppress the development of various immune cells and pro-inflammatory cytokines, displayed in Figure 2 (150, with kind permission by Elsevier,153).

Identification: In our study we identified regulatory B cells as $CD19^+$, $CD24^+$ and $CD38^+$.

1.3 Cytokines

Cytokines are a heterogeneous group of different proteins that are responsible for intercellular communication and essential for a functioning immune system (154). Etymologically its Greek components mean “cell movement”. The small peptides are between 8 and 30 Kilo Dalton (kDa) and can act as autocrine, endocrine and paracrine agents which leads to a certain overlap with hormones (155). Cytokines can be roughly subcategorized into Colony-stimulating factors (CSF), Interferons, Interleukins and Tumor Necrosis Factors (18). Table 2 provides a selection of the relevant cytokines investigated in

this study. Note that the below mentioned Granzyme B is technically not a cytokine but due to its role in cytokine release and also as an important effector molecule of CTLs, it can be assumed that measurement of Granzyme B levels gives a better understanding of a healthy human immune system.

Table 2 Cytokine overview (18): ↑= increase, ↓= decrease

Cytokine	Producer	Function
Granulocyte-macrophage colony stimulating factor	Macrophages, T cells	Increase of neutrophil development and proliferation
Granzyme B	Cytotoxic lymphocytes, natural killer cells	Initiation of apoptosis via caspases, release of cytochrome c from outer mitochondria membrane
Interferon alpha	Dendritic cells, leukocytes	MHC I expression↑
Interferon gamma	Intraepithelial lymphocytes, natural killer cells, neutrophils, T cells	Enhanced expression of MHC on cell surface, Ig class switch, macrophage activation, Th2 and Th17 suppression
Interferon gamma-induced Protein 10 (IP-10)	Endothelial cells, fibroblasts, monocytes	Induces chemotaxis of dendritic cells, natural killer cells, macrophages and T cells, promotes the adhesion of T cells to endothelium
Interleukin 2	T cells	T cell proliferation↑ and differentiation↑, Treg maintenance
Interleukin 4	Mast cells, T cells	B cell activation, IgE switch, promotes Th2 differentiation
Interleukin 5	Mast cells, T cells	Differentiation↑ and proliferation↑ of eosinophils
Interleukin 8	Endothelial and epithelial cells, fibroblasts, monocytes	Neutrophil's chemotaxis↑, angiogenesis
Interleukin 9	T cells	Augmentation of mast cell activity↑, stimulates Th2 cells
Interleukin 10	B cells, dendritic cells, Macrophages, T cells	Moderation of immune response, suppression of macrophage function,

Interleukin 13	T cells	B cell differentiation↑ and proliferation↑, inhibits production of inflammatory cytokines and Th1 cell differentiation, induces allergic diseases and asthma
Interleukin 17A	$CD8^+$ T cells, natural killer cells, neutrophils, Th17	Pro-inflammatory function, production↑ of cytokines and antimicrobial peptides by epithelial tissue, endothelia and fibroblasts
Interleukin 18	Activated macrophages	IFN-γ production↑ by T cells and natural killer cells, Th1 proliferation↑
Interleukin 22	Natural killer cells, neutrophils, Th17	Production↑ of antimicrobial peptides, acute-phase proteins and pro-inflammatory agents
Interleukin 23	Dendritic cells, macrophages	Proliferation↑ of Th17 memory cells, IFN-γ production↑
Interleukin 1 receptor antagonist (IL1RA)	Macrophages, neutrophils	Interleukin 1 activity↓
Tumor necrosis factor alpha	Macrophages, natural killer cells, T cells	Stimulates inflammation, activation of endothelia, together with CTL's Fas-L co-stimulator of apoptosis

1.4 Cell surface molecules

The cell surface is an important factor of immunological research. CDs or chemokine receptors can be targeted with antibodies to either identify or stimulate a cell. This subsection provides an overview of the relevant chemokine receptors and clusters of differentiation.

1.4.1 Chemokine receptors

Concerning the chemokines, which belong to the group of cytokines, there are about 50 different proteins, not all of them being selective to only one chemokine receptor. Table 2

provides an overview of the mentioned chemokine receptors and CDs in this study and gives additional information.

Members of the CXC chemokine family can be divided based on the three amino acids before the first cysteine rest. If these acids are Glutamic acid, Leucine and Arginine the chemokine is ELR^+ and chemotactic for granulocytes (18). ELR represents the 1-letter abbreviation of the three amino acids. When the amino acids are configured differently the chemokine is ELR^- and chemotactic for lymphocytes (18).

Table 3 Relevant chemokines and their receptors (18)

Chemokine receptor	Chemokine	Target cell
CXCR3	Mig (ELR^-) IP-10 (ELR^-) I-TAC (ELR^-)	Mig, IP-10 and I-TAC: Activated T cells, B cells, endothelial cells, dendritic cells, natural killer cells
CXCR4	SDF-1α/β (ELR^-)	Naïve activated T cells, B cells, plasma cells, immature and mature dendritic cells
CXCR7	SDF-1α/β (ELR^-)	Naïve activated T cells, B cells, plasma cells, immature and mature dendritic cells
CCR3	Eotaxin Eotaxin-2/MPIF-2 Eotaxin-3 HCC-1 MCP-3 MCP-4 MIP-5/HCC-2	Eotaxin: Eosinophil, basophil, mast cell, Th2 Eotaxin-2/MPIF-2: Eosinophils, basophils, T cells Eotaxin-3: Eosinophils, basophils, fibroblasts HCC-1: Monocytes MCP-3: Th1, Th2, monocytes, eosinophils, basophils, immature dendritic cells, natural killer cells MCP-4: Th1, Th2, monocytes, eosinophils, basophils, dendritic cells MIP-5/HCC-2: T cells, monocytes, eosinophils, dendritic cells

	RANTES	RANTES: Monocytes, T cells, natural killer cells, basophils, eosinophils, immature dendritic cells
CCR4	TARC MDC	TARC: Th1, Th2, immature dendritic cells, thymocytes, Tregs MDC: Immature dendritic cells, natural killer cells, Th1, Th2, thymocytes, endothelial cells, monocytes, Tregs
CCR5	MIP-1α/LD78 MIP-1β RANTES MCP-2 MCP-3 Eotaxin	MIP-1α/LD78: Monocytes, Th1, Th2, natural killer cells, basophils, immature dendritic cells, eosinophils, neutrophils, astrocytes, fibroblast, osteoclasts MIP-1β: Monocytes, Th1, Th2, natural killer cells, basophils, immature dendritic cells, eosinophils, B cells RANTES: Monocytes, T cells, natural killer cells, basophils, eosinophils, immature dendritic cells MCP-2: Th1, Th2, monocytes, eosinophils, basophils, immature dendritic cells, natural killer cells MCP-3: Th1, Th2, monocytes, eosinophils, basophils, immature dendritic cells, natural killer cells Eotaxin: Eosinophil, basophil, mast cell, Th2 Eotaxin-2/MPIF-2: Eosinophils, basophils, T cells HCC-1: Monocytes

	HCC-1 HCC-4/LEC	HCC-4/LEC: Monocytes, T cells, natural killer cells, immature dendritic cells
CCR6	MIP-3α/LARC	MIP-3α/LARC: T cells, immature dendritic cells, activated B cells, natural killer cells, GALT
CCR7	MIP-3β/ELC 6Ckine/SLC	MIP-3β/ELC: Naïve T cells, mature dendritic cells, B cells 6Ckine/SLC: Naïve T cells, B cells, thymocytes, natural killer cells, mature dendritic cells
CCR8	HCC-4/LEC TARC	HCC-4/LEC: Monocytes, T cells, natural killer cells, immature dendritic cells TARC: Th1, Th2, immature dendritic cells, thymocytes, Tregs

1.4.2 Cluster of Differentiation

To date, 371 Clusters of Differentiation have been discovered and named. Depending on the CD and the cell type, they are expressed permanently or transiently.
Table 4 provides an overview of the mentioned clusters of differentiation in this study and gives additional information.

Table 4 Relevant Clusters of Differentiation (18)

CD (Alias)	Expressed on	Function
CD3	T cells, thymocytes, natural killer cells	Expression of and signal transduction via TCR
CD4	T helper cells,	Co-receptor for MHC II

CD8	Cytotoxic T cells	Co-receptor for MHC I
CD19	B cells	Co-B cell receptor together with CD21 and CD81
CD21 (CR2)	Mature B cells, dendritic cells	Co-B cell receptor together with CD19 and CD81, receptor for C3d
CD24	B cells, granulocytes	Sialoglycoprotein
CD25	Activated T cells, B cells	Interleukin 2 receptor, α chain
CD27	T cells, natural killer cells	Co-stimulator for T and B cells, binds CD70
CD28	T cell subsets, activated B cells	Naïve T cell activation, second signal receptor, binds B7.1 and B7.2
CD38	Activated T cells, germinal center B cells, plasma cells	Increases B cell proliferation
CD40	B cells, macrophages, dendritic cells	Binds CD40-Ligand (CD154), second signal receptor for TD B cell activation, promoting further differentiation and proliferation
CD45	All hematopoietic cells	Augmentation of B and T cell's antigen receptor signalling
CD45RA	B cells, T cell subsets	Isoform of CD45

CD45RO	T cell subsets B cell subsets	Isoform of CD45
CD70	Activated T and B cells, macrophages	Ligand to CD27, co-stimulator for T and B cells
CD80 (B7.1)	B cell subsets	Co-stimulator with CD28 (ligand), second signal for T cell activation
CD81	Lymphocytes	Co-B cell receptor together with CD19 and CD21
CD86 (B7.2)	Monocytes, activated B cells, dendritic cells	Co-stimulator with CD28 (ligand), second signal for T cell activation
CD103	Intraepithelial lymphocytes	Integrin
CD124 (IL4-R)	Mature B and T cells	Interleukin 4 receptor
CD127 (IL-7R)	Mature T cells	Interleukin 7 receptor
CD152 (Cytotoxic T-lymphocyte associated protein, CTLA-4)	Activated T cells	Receptor for B7.1 and B7.2
CD154 (CD40L)	Activated $CD4^+$ T cells	CD40-Ligand, second signal for TD B cell activation, differentiation and proliferation
CD183 (CXCR3)	Th1 cells	Chemokine receptor, binds INP10 and MIG
CD194 (CCR4)	Th2, Th17, Tregs, CTLs, monocytes, B cells	Chemokine receptor for CCL17 and CCL22
CD196 (CCR6)	Th17, natural killer cells, Tregs	Chemokine receptor for CCL20 and CCL21
CD197 (CCR7)	Activated B and T cells	Chemokine receptor for MIP-3β

CD212 (IL-12R)	Activated $CD4^+$ and $CD8^+$, natural killer cells	Interleukin 12 receptor β chain
CD218a (IL-18Ra)	Macrophages, neutrophils, natural killer cells, T cells	Trigger of cytotoxic response

2 Methods

2.1 Recruitment

In cooperation with the Styrian health insurance (Steiermärkische Gebietskrankenkasse) healthy persons, who visited the insurance's outpatient clinic in order to participate in the Austrian health examination program ("Gesundenuntersuchung"), were asked to join the study. Before giving written consent they were informed about the study, the extent of their participation and possible side effects of a blood draw by one of the out-patient-clinic's doctors (see Appendix I: Patient information and consent form, in German).

The participants then gave written informed consent to a blood draw of 43ml of peripheral venous blood (5x 8ml vacutainers coated with lithium heparin and 1x 3ml vacutainer coated with ethylenediaminetetraacetic acid (EDTA)). A questionnaire was filled out by the outpatient clinic's doctors in cooperation with the test persons in question to assess their health status (see Appendix II Case report form, in German). Neoplasia, acute or in remission, infectious disease, acute or chronic, current pregnancy, severe anemia (Hemoglobin (Hb) <9mg/dl), autoimmune disease and acute or chronic diseases associated with organ damage led to exclusion. One hundred and thirty two persons consented to participate in the prospective study. None of the test persons fulfilled the exclusion criteria. Due to problems with the blood draw because of insufficient peripheral veins and hemolytic samples, three test persons were excluded from the sample group. One test person's blood tests showed significant aberrations, which suggested a hematological disease and led to exclusion and transferal to a hematologist. One hundred and twenty eight test persons were included in the study. Table 5 summarizes the patient characteristics. Demographic and anamnestic data was gathered from the test persons (age, gender, body mass index (BMI) and smoking status (yes/no)).

The blood samples were delivered to the immunology laboratory for processing and preparation, located at the state and university hospital Graz (LKH-Universitätsklinikum Graz). On site, the current level of C-reactive protein (CRP) in the blood samples was measured. Massive elevated levels led to exclusion due to possible systemic inflammation.

Table 5 Demographic data of test persons

Number of test persons	132
Included	128
Age (mean value)	51 (standard deviation (SD) 15)
Sex (female/male)	56/72
Inclusion criteria	- Legal age
Exclusion criteria	- Neoplasia, acute or in remission - Infectious disease, acute or chronic - Current pregnancy - Severe anemia (Hb <9mg/dl) - Autoimmune disease - Acute or chronic diseases associated with organ damage - Elevated CRP levels

2.2 Consent from the ethical review committee

Permission to conduct the study was obtained from the Medical University of Graz' ethical review committee on September 12, 2014 (Number: 26-559 ex 13/14, see Appendix III Consent by ethical review committee, in German)

2.3 FACS

Flow cytometry is a frequently used technique in immunology to count cells by letting them pass through a laser beam individually (18). When the cytometer is not only able to count but also to separate and sort the cells, the method is called fluorescence activated cell sorting (18). Using FACS, a large amount of cells can be subcategorized into their distinctive subsets due to a high throughput, which is extremely helpful when dealing with a heterogeneous group of cells like lymphocytes (156). To signal the cytometer which cell belongs to which group, the cells are marked with monoclonal antibodies against surface or intracellular proteins (see 2.4 Antibody staining) (18). Attached to the antibodies are fluorescent dyes

that are excited by laser beams and detected by sensors. To guarantee that there is no overlay of signals, the cell suspension is diluted with saline or staining solutions and is subsequently forced through a small nozzle which creates a fine stream of liquid in which the cells pass the laser beam one at a time (19). Every time a cell passes the beam, it scatters the laser light. If this cell is also marked with a fluorescent antibody, the dye emits fluorescence. Sensors detect both the scattered laser light as well as the emitted fluorescence. The front scatter (FSC) detector gives information about the size of the passing cell while the side scatter (SSC) detector tells about the cell's granularity (157). Other sensors detect fluorescence and can identify the binding of a distinctive antibody and therefore the expression of a selective surface protein or intracellular protein (17). Results are displayed in histogram (1 parameter) or dot plots (2 or more parameters) (18). For our study we used a FACS Canto II (manufactured by Becton Dickinson, Franklin Lakes, NJ, USA).

2.3.1 Analysis of flow cytometry data

The processing, analysis and gating of the raw data gathered from FACS was done with the software FlowJo Version 10.4.1 (manufactured by FlowJo LLC, Ashland, OR, USA) and BD FACS Diva (BD). The measurement of the cytokine levels was performed using xPONENT Software (Luminex Corporation, Austin, TX, USA).

2.4 Antibody staining

For antibody staining different customized antibody cocktails manufactured by Miltenyi Biotech (Bergisch Gladbach, NRW, Germany) and a Ki67 antibody stain, produced by BD, were used. Each antibody cocktail with their containing antibodies are listed separately below. The staining was performed on the same day as the blood draw and was done according to our standard operating procedures. Over the course of the study, the same investigators performed staining and FACS analysis. Internal quality control was assured by daily calibration of the FACS Canto II and the Beckman automated cell coulter.

2.4.1 T cell panel 1

The T cell panel 1 (T1) was used to perform the staining of both the surface and the intracellular compartment of the immune cells. The blood samples were taken from the EDTA coated tube. Due to the use of antibodies marked with fluorescent dyes, the incubation took place in darkness to prevent the bleaching of the fluorochromes. It was also performed at room temperature.

After the surface staining we used FoxP3 staining buffer (Manufacturer: e-Bioscience, San Diego, CA, USA) to fixate the cellular structures and permeabilize the cell membrane to enable the antibodies to reach the subcellular compartments. After another cycle of incubation and washing with permeabilization buffer, which is used to prevent the cell membrane from degrading too much, we stained the cells with Ki67 antibodies. Ki67 is a protein, which can be found in proliferating cells and is therefore used as a marker of proliferation. The cells were washed and centrifugalized again to remove all unnecessary cells, e.g. erythrocytes and thrombocytes. After resuspensation with staining buffer, the sample was analyzed with the FACS machine.

SOP for T1 panel:

1. 200 µl blood from EDTA tube
2. 10 µl T1 panel antibody cocktail, see Table 6 for components
3. Incubation for 15 minutes at room temperature and in darkness
4. Preparation of Fixation/Permeabilization solution, consisting in a ratio of 1:4 of 1 ml concentrate and 3ml diluent (both manufactured by e-Bioscience)
5. 1ml of Fixation/Permeabilization solution
6. 30 minutes of incubation at 4°C
7. 2x washing with 1ml permeabilization buffer (Flow Cytometry Staining Buffer Solution, e-Bioscience) and centrifugation 300-400g for 5 minutes
8. Resuspend with 100µl of permeabilization buffer
9. 10µl of Ki67 PercP-Vio700 (BD)
10. Incubation for 30 minutes at room temperature and in darkness
11. 2x washing with 1ml permeabilization buffer and centrifugation 300-400g for 5 minutes
12. 100µl of staining buffer (e-Bioscience)
13. Analysis with FACS CANTO II (BD)

Table 6 **Antibody cocktail of T1 panel**

Antibody	Fluorochrome
CD3	APC Vio 770
CD4	VIT4 Vio Blue
CD8	VioGreen
CD183/CXCR3	PE
CD196/CCR6	PE Vio 700
CD194/CCR4	APC
CD38	FITC

2.4.2 T cell panel 2

The T cell panel 2 (T2) antibody cocktail was used to stain the cell surfaces. The blood sample was again taken from the EDTA tube. Afterwards FACS lysis solution (BD) was used to lyse the erythrocytes. After centrifugation and discarding the supernatant the remaining leukocytes were washed and centrifuged twice and resuspended with Cellwash (BD) to prepare for FACS analysis.

SOP for T2 panel:

1. 200 µl blood from EDTA tube
2. 10 µl T2 panel antibody cocktail, see Table 7 for components
3. Incubation for 15 minutes at room temperature and in darkness
4. 2 ml of FACS Lysis solution
5. Incubation for 15 minutes at room temperature and in darkness
6. Centrifugation for 5 minutes at 300 g
7. Discard supernatant, resuspend with 2 ml Cellwash
8. Centrifugation for 5 minutes at 300 g
9. Discard supernatant, resuspend with 2 ml Cellwash
10. Centrifugation for 5 minutes at 300 g
11. Discard supernatant, resuspend with 150 µl Cellwash
12. Analysis with FACS CANTO II

Table 7 **Antibody cocktail of T2 panel**

Antibody	Fluorochrome
CD3	APC-Vio770
CD4 (Vit4)	PerCP-Vio770
CD8	VioGreen
CD197/CCR7	APC
CD127	PE-Vio770
CD28	PE
CD25	VioBright-FITC
CD45RA	VioBlue

2.4.3 B cell panel

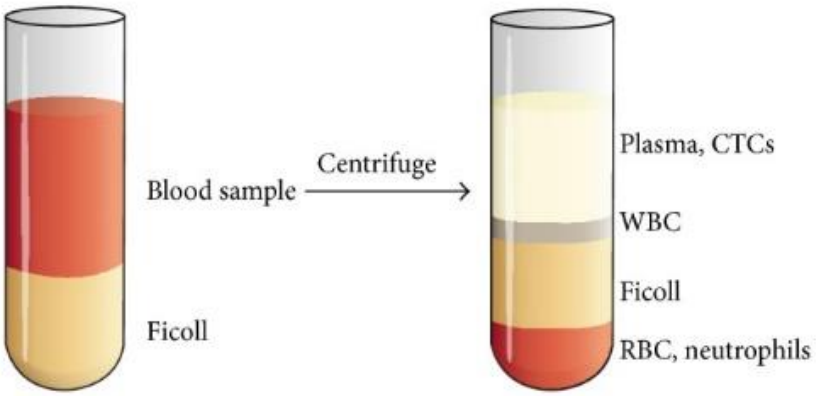


Figure 3 Density centrifugation separation method (158) With the help of Ficoll/Histopaque the cellular components of the blood sample can be separated during centrifugation according to their density. CTC=Circulating tumor cells, WBC=PBMC.

While the number of T cells in peripheral venous blood samples is high enough to stain in a whole blood sample, the share of B cells in the group of lymphocytes is significantly lower. In order to investigate B cells, it is therefore necessary to isolate the mononuclear cells (lymphocytes and monocytes) from other blood components like erythrocytes or thrombocytes. To isolate the peripheral blood mononuclear cells (PBMCs) the density-gradient fractionation was used, which is displayed in Figure 3 (158, Creative Commons Attribution License). This method uses the blood component's differences in density by applying a step gradient (e.g. Ficoll or Histopaque, both tradenames for highly branched copolymer of hydrophilic polysaccharide) underneath the peripheral blood (18). In this study we used Histopaque (H1077 Hybri-Max; Sigma-Aldrich, St. Louis, MO, USA), whose density is in between the densities of red blood cells (RBC) and the PBMCs/plasma. Histopaque, in equal parts to the amount of peripheral blood, was filled to a 50ml tube. The

peripheral blood was diluted with phosphate buffered saline (PBS) and carefully added to the tube at a ratio of 1:1. After centrifugation, the RBCs and the granulocytes have traveled to the bottom of the tube (18). The next layer is the Histopaque, on top are the PBMCs and the top layer consists of blood plasma and thrombocytes. The PBMCs are extracted carefully and can be counted with an automated cell counter (Beckmann Coulter, CA, USA). 10^6 PBMCs were diluted with PBS and then stained with the antibody cocktail. After incubation, washing and centrifugation the cells were analyzed with the FACS CANTO II.

SOP for B cell panel:

1. Dilution of the blood of two lithium heparin coated tubes with PBS at a ratio 1:2,5 , pH 7,2-7,3, manufactured by the pharmacy of the State and University Hospital Graz)
2. Add Histopaque to a 50 ml tube (the amount of Histopaque equals the amount of blood in a ratio 1:1)
3. Gradually apply the diluted blood as an overlay to the Histopaque without mixing the two phases
4. Centrifugation at 400 g for 30 minutes
5. Remove PBMCs with pipette and add it to 12ml tube
6. Add 12 ml of PBS
7. Centrifugation at 250 g for 10 minutes
8. Discard the supernatant and refill with 12 ml PBS
9. Centrifugation at 250 g for 10 minutes
10. Discard the supernatant and resuspend with 300 µl PBS
11. Counting of cells with automated cell counter
12. Extract 10^6 PBMCs and dilute with 100 µl PBS
13. 10 µl B cell antibody cocktail, see Table 8 for components
14. Incubation for 15 minutes in darkness
15. 2x washing with 2 ml Cellwash and centrifugation at 300 g for 5 minutes
16. 150 µl Cellwash
17. Analysis with FACS CANTO II

Table 8 **Antibody cocktail of B cell panel**

Antibody	Fluorochrome
CD19	Vio Green
IgD	Vio Blue
CD24	PerCP-Vio700
CD27	APC
CD38	FITC
CD86	PE-Vio770
CD21	APC-Vio770
IgM	PE

2.4.4 B cell and T cell stimulation

In preparation for the lymphocyte stimulation, PBMCs were gathered using the density gradient centrifugation method described in the section above. A cell culture was created in 96 wells plates (Greiner, Kremsmünster, UA, Austria) with Roswell Park Memorial Institute 1640 medium (RPMI 1640, manufactured by Invitrogen/Gibco (Life Technologies/Thermo Fisher Scientific), Carlsbad, CA, USA) as cell culture medium. The medium contained 10% fetal bovine serum (FBS, Invitrogen/Gibco), Penicillin-Streptomycin (Invitrogen/Gibco) and L-Glutamine (Invitrogen/Gibco).

The T cell stimulation was performed at 37°C and 5% CO_2 for 72h, using anti-CD3/anti-CD28 coated beads (Invitrogen/Gibco), 5 mg or 10 mg of Concanavalin A (ConA, Sigma-Aldrich). The CD3/CD28 antibodies, which are bound to the beads, imitate the natural process of T cell activation through the APCs (159–161). ConA is a lectin extracted from the jack-bean and is used as a mitogen (162,163).

The B cells were also stimulated at 37°C and 5 % CO_2 but for the duration of 7 days using 0.25 µM or 0.125 µM of ODN2395 (Miltenyi). ODN2395 is a class C oligodeoxynucleotide that acts as an agonist for the humane and murine TLR9 (164).

In addition to the mitogens, CellTrace™ Violet (Thermo Fisher Scientific, Waltham, MA, USA) was added to both B and T cells before cultivation to analyze the induced lymphocyte proliferation capacity via FACS. CellTrace™ is a non-fluorescent dye that is incubated together with the lymphocytes. Once it reached the cytosol, the dye becomes fluorescent and

binds covalently to cellular proteins. With every cell division the dye-binding proteins are inherited to the daughter cells, while the first generation contains approximately 50% of the proteins each, the second 25%, et cetera. Through FACS analysis, the number of generations can be detected.

After cultivation, T and B cells were stained each with premade antibodies (Miltenyi), as illustrated in Table 9, and incubated for 15 minutes in darkness. Then they were washed with 1 ml Cellwash, resuspendend with 150 µl Cellwash and analyzed.

Table 9 Antibodies for B and T cell stimulation

B cell antibodies	Fluorochrome	T cell antibodies	Fluorochrome
CD19	APC-Vio 700	CD3	APC-Vio 770
CD38	FITC	CD4	Vio Bright FITC
IgD	PE-Vio 770	CD8	PerCP-Vio 700
CD27	APC		
IgM	PE		

2.4.5 Cytokines

In this study, we measured levels of GM-CSF, Granzyme B, Interferon gamma, Interleukin 2, Interleukin 4, Interleukin 5, Interleukin 9, Interleukin 10, Interleukin 13, Interleukin 17A, Interleukin 22, and Interleukin 23 gathered from the supernatant of stimulated T- cells (see 2.4.4 B cell and T cell stimulation). We also measured Interleukin 5, Interleukin 8, Interleukin 17A, Interleukin 18, Interleukin 1 receptor antagonist (IL1RA), TNF-alpha, Interferon alpha, Interferon gamma and Interferon gamma-induced protein 10 (IP-10) from the test person's non-stimulated plasma (see 1.3. Cytokines and Table 2). To analyze and measure the levels of the cytokines, the multiplex protein analysis MAGPIX® System was used. This system uses magnetic beads/microspheres with specific antibodies on their surface, which are added to the samples. Every bead is color coded with a unique spectral signature. A fluorochrome-labeled detection antibody is added to indicate the number of molecules bound to each bead. Light-emitting diodes excite the fluorochromes. A camera detects the signals, corresponding to each kind of bead and the number of molecules bound to it (165).

2.5 Statistical data analysis

The statistical analysis was performed using SPSS version 23 (IBM, Armonk, NY, USA) run on Windows 7 and Windows 10 (both Microsoft, Redmond, WA, USA).

2.5.1 Reference intervals

Two factors need to be considered when trying to establish reference intervals.
The first is the sample size, which should be at least n=120, according to the National Committee for Clinical Laboratory Standards (NCCLS) (166,167). A sample of minimum 120 allows the calculation of the 90% confidence intervals for the respective endpoints of the reference ranges and gives RIs a certain degree of validity (167). It should be noted, that a smaller sample size does not automatically contradict the estimation of RIs, but it might not be possible to calculate valid confidence intervals (167).
The second factor is the possible influence of outliers, especially in RIs that are estimated from small samples and in a non-parametric method (168).
Taking the two factors mentioned above into account, our strategy consists of multiple steps that are based on the work of Horn and Pesce (2003) (167) and the recommendations of the NCCLS (166) and the International Federation of Clinical Chemistry (IFCC) (169).

The first step was the logarithmic transformation of the data. In the next step, we tested the samples for outliers. The NCCLS recommend the application of Dixon's criteria (166,170), which calculates the ratio between D (difference between most extreme and adjacent value) and R (range minimum value to maximum value). From this can be derived that:

$$\frac{D}{R} = < 0.33 \; = No\ elimination\ of\ Outlier$$

and in return

$$\frac{D}{R} = > 0.33 \; = Elimination\ of\ Outlier$$

When there is more than one possible outlier, the NCCLS recommends to test the least extreme one as the only outlier and, if above 0.33, all more extreme outliers are eliminated

as well (166,167). Linnet (1987) argues, that the critical value needs to be adjusted according to the sample size and the form of distribution (171). Our study would therefore apply critical values of 0.26 for a Gaussian and 0.53 for a skewed distribution. In three cases Dixon's adjusted criteria was used to establish RI.

However, Horn and Pesce (2003) as well as the IFCC chose a different approach, which we adapted for our study, because it proves to be more independent from the issue of distribution and is able to handle a larger amount of possible outliers (167,169). According to their method, we computed boundaries of the RI by calculating the lower (Q_1) and upper quartile (Q_3) and the interquartile range (IQR).

$$IQR = Q3 - Q1$$
$$Lower\ boundary = Q1 - 1.5 \times IQR$$
$$Upper\ boundary = Q3 + 1.5 \times IQR$$

Any data lying beyond the lower or upper boundary was identified as an outlier and excluded from the sample (167,169) before we tested for Gaussian distribution using the Kolmogorov-Smirnov and Shapiro-Wilks test.

In case of (approximate) normal distribution, the reference interval was determined as

$$RI = mean \pm 1.96 \times SD$$

In the next step we calculated the 90% confidence interval (CI) of the lower and upper reference value (RV) using the standard error (SE) of the respective limit (171):

$$CI = RV \pm 1.645 \times SE$$

with SE defined as:

$$SE = \sqrt{\frac{SD^2}{n} + 1.96^2 \frac{SD^2}{2n}}$$

If the sample was skewed, we used a non-parametric technique to define the lower and upper limits of the RI by ranking the data and determine the 2.5 and 97.5 percentile (167,169). The non-parametric approach to define 90% CI for the RVs is possible if the sample size is ≥119

(172). If that proved to be the case, the data was again ranked, with the lowest number being rank number one and the highest being rank number n (n = sample size). In a sample size between 119 and 132, the CI of the lower RV was defined as the data with rank numbers 1 and 7. For the upper RV, 1 and 7 were each subtracted from n+1, equalizing the respective rank numbers of the upper CI (166,172). Figure 4 summarizes our strategy for establishing the reference intervals.

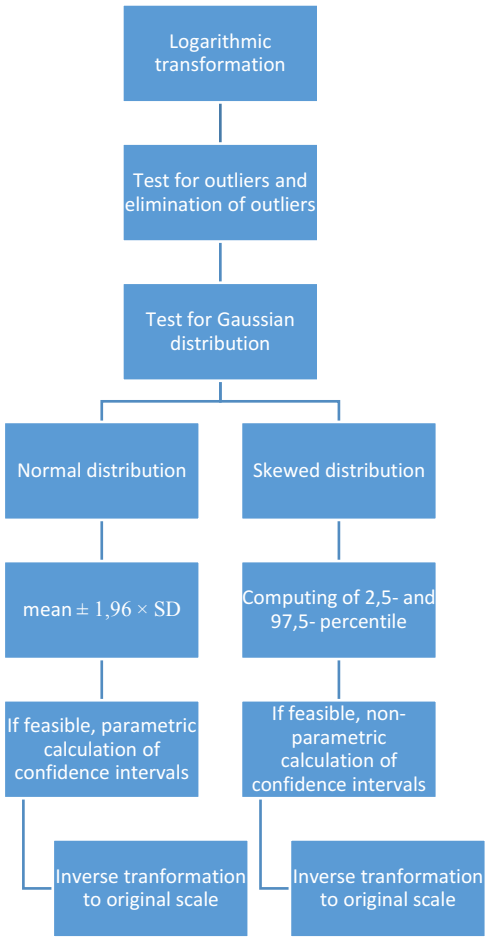


Figure 4 Strategy for the estimation of reference intervals

2.5.2 Correlation

Pearson's correlation coefficient was used to calculate correlations in normal distributed data while Spearman's Rho was used to test for correlations within skewed data. To investigate mean differences regarding gender and smoking status the Mann-Whitney-U-test was used.

3 Results

3.1 T cell panel 1

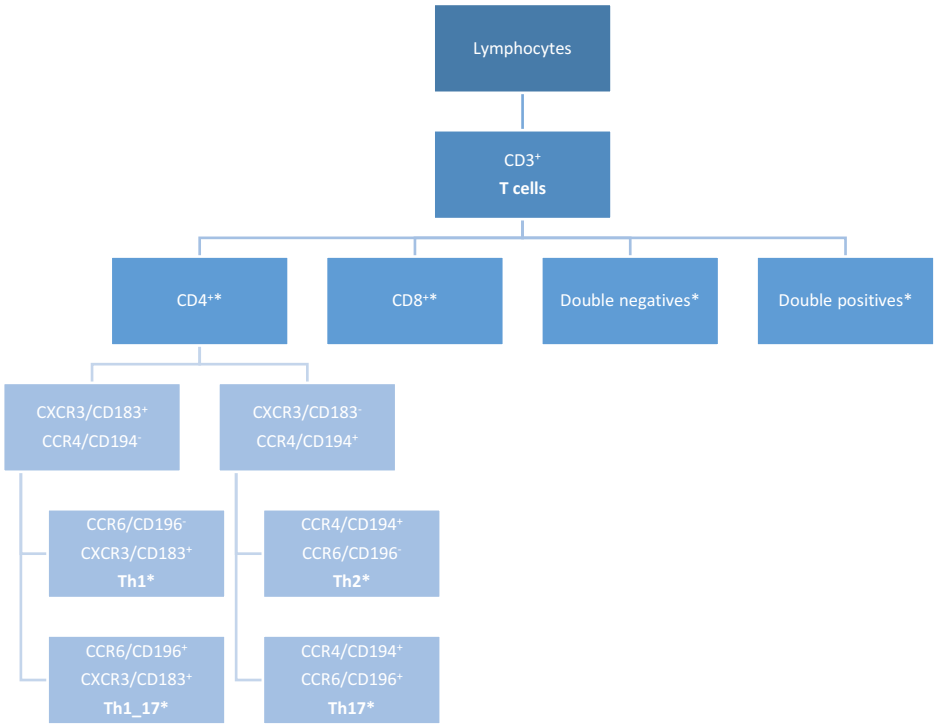
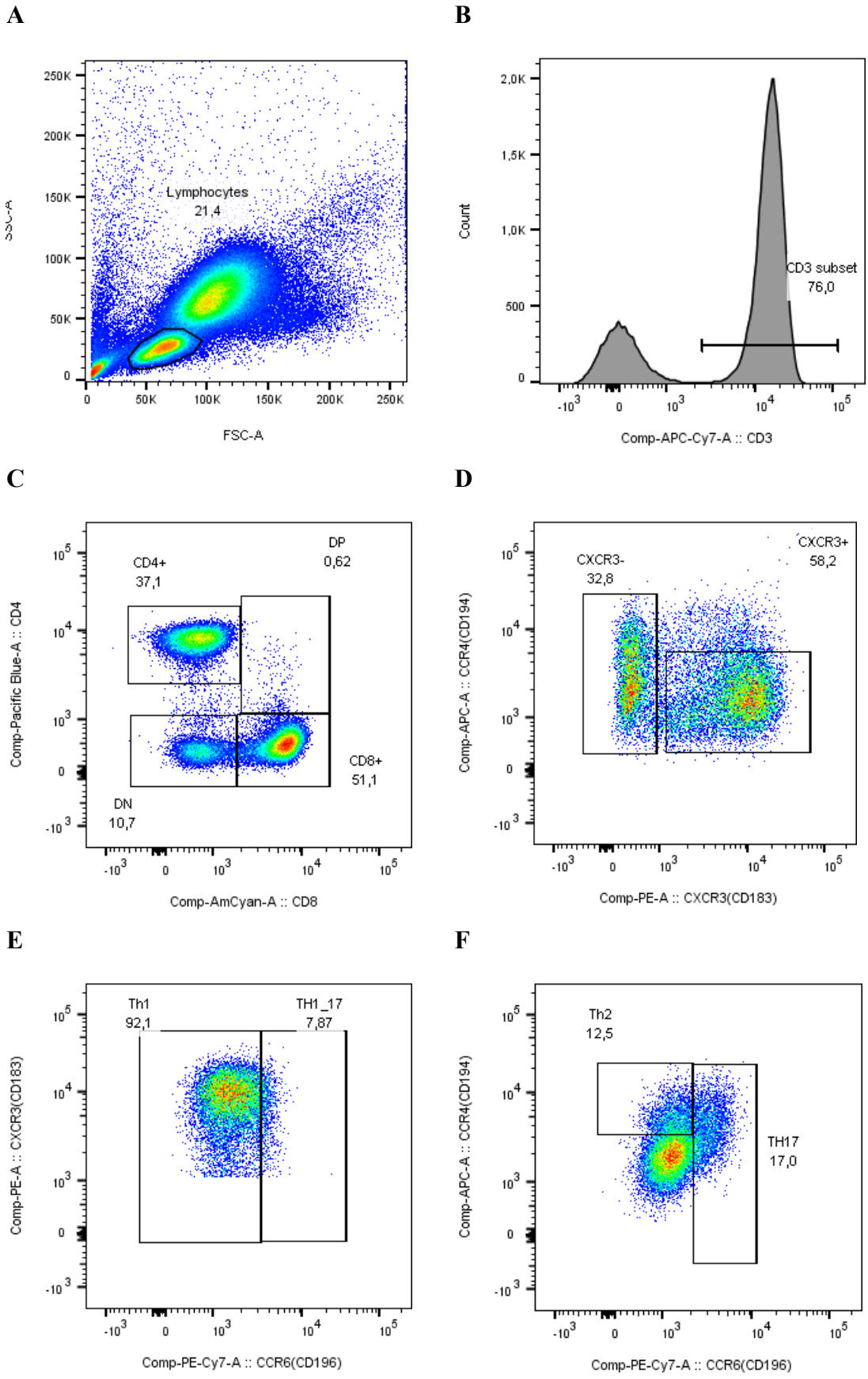


Figure 5 Gating strategy for the T1 panel. *= Gating for activation and proliferation using CD38 and Ki67 was performed

In the first step we gated the lymphocyte population from the remaining cells based on size and granularity. With CD3 we determined the T-lymphocytes. The T cells were then subcategorized into $CD4^+$, $CD8^+$, $CD4^+$ and $CD8^+$ or double positives (DPs) and $CD4^-$ and $CD8^-$ double negatives (DNs). With CXCR3 the helper cells were further divided into CXCR3 positives and negatives. The $CXCR3^-$ cells were classified as Th2 ($CCR4^+$, $CCR6^-$) and Th17 ($CCR6^+$). The $CXCR3^+$ cells were defined as Th1 ($CXCR3^+$, $CCR6^-$) and as Th1_17 ($CXCR3^+$, $CCR6^+$). In a last step, $CD4^+$, $CD8^+$, DN, DP, Th1, Th1_17, Th2 and Th17 were gated with CD38 as a marker for activated cells and Ki67 as a proliferation marker. Figure 5 and 6 summarize the gating strategy.


A
Lymphocytes
21,4
FSC-A
B
Count
CD3 subset
76,0
Comp-APC-Cy7-A :: CD3
C
CD4+
37,1
DP
0,62
CD8+
51,1
DN
10,7
Comp-Pacific Blue-A :: CD4
Comp-AmCyan-A :: CD8
D
CXCR3-
32,8
CXCR3+
58,2
Comp-APC-A :: CCR4(CD194)
Comp-PE-A :: CXCR3(CD183)
E
Th1
92,1
TH1_17
7,87
Comp-PE-A :: CXCR3(CD183)
Comp-PE-Cy7-A :: CCR6(CD196)
F
Th2
12,5
TH17
17,0
Comp-APC-A :: CCR4(CD194)
Comp-PE-Cy7-A :: CCR6(CD196)

G

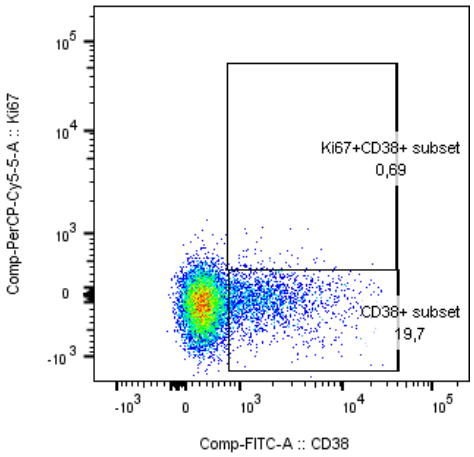


Figure 6 Representative dot plots of the T1 panel gating strategy
A: Determination of lymphocytes based on cell size (FSC) and cell granularity (SSC)
B: Gating of T cells via anti-CD3 antibody staining
C: T cell subclass gating using anti-CD4 and anti-CD8 antibodies
D: T helper cells are subcategorized based on CXCR3
E-F: $CXCR3^+$ cells are divided into Th1 and Th1_17, $CXCR3^-$ cells are divided into Th2 and Th17
G: Cell activity and proliferation from CD4,Th1,Th2,Th1_17,Th17,CD8,DP and DN were determined using CD38 and Ki67

3.1.1 T cell panel 1 reference intervals

Tables 10 provides a summary of the T1 panel reference intervals. The numbers were rounded to zero decimal place, if the width of the confidence intervals still contained the reference range. The table shows the total sample size, the sample size after outlier elimination, the percentage (%) and absolute (abs) RI, the method of calculation (parametric (par) or non-parametric (npar)), the 90% confidence intervals for the lower and upper limits of the reference range and the number of outliers. If the population was not normally distributed and the sample size was below 119, the calculation of the CIs was mathematically not possible and was therefore marked with an X.

Table 10 T cell panel 1 reference intervals

Subset	**n** (total)	**n** (%/abs)	**RI** (%)	**90%-CI** (%)	**RI** (cells/µl)	**CI** (cells/µl)	**Parametric / non-parametric** (%/abs)	**Outlier** (%/abs)
T1 $CD3^+$	128	119 / 128	42 - 79	38 – 45 / 76 - 83	645 – 2355	630 – 691 / 2137 - 2511	npar / npar	9 / 0

T1 $CD4^+$	128	125 / 128	48 - 86	47 – 50 / 81 - 91	389 – 1653	309 – 447 / 1445 – 1905	npar / npar	3 / 0
T1 $CD8^+$	128	123 / 125	15 – 45	13 – 15,1 / 40 - 51	126 – 754	91 – 138 / 589 - 794	npar / npar	5 / 3
Th1	128	125 /125	16,9-51,5 %	16,84-17 / 51,45-51,72	108,01-526,05	97,48-119,44 / 475,13-582,19	npar / par	3 / 3
Th1/$CD38^+$	128	125 / 120	8,8-37, %	8,01-9,65 / 34,28-41,3	18,06-108,72	16-20,38 / 96,85-122,05	npar / par	3 / 8
Th1/$CD38^+$ $KI67^+$	128	124 / 124	<1,5 %	1,11 – 1,57	<3,74	2,71 – 4,49	npar / npar	4 / 4
Th1_17	128	126 / 128	<11,24 %	6,24 – 12,48	<92,06	65,06 – 101,32	npar / npar	2 / none
Th1_17 /$CD38^+$	128	120 / 128	4,35 – 37,90 %	3,77 – 5,01 / 32,89 – 43,67	<15,26	9,47 – 20,87	par / npar	8 / none
Th1_17 /$CD38^+$ $KI67^+$	128	114 / 114	<0,6 %	X	<0,13	X	npar / npar	14 / 14
Th2	128	121 / 123	2,2 – 12, 6 %	0,5 – 3,12 / 9,71 – 14,71	17,34 – 127,65	15,2 – 19,73 / 104,71 / 145,24	npar / par	7 / 5
Th2/$CD38^+$	128	126 / 120	6,2 – 39 %	6,13 – 6,48 / 38,83 – 39,11	2,33 – 17,22	1,98 – 2,72 / 15,3 – 19,36	par / par	2 / 8
Th2/$CD38^+$ $KI67^+$	128	111 / 113	<0,8%	X	<0,37	X	npar / npar	17 / 15
Th17	128	127 / 125	1,1 – 20 %	0,86 – 1,26 / 16,54 – 24,06	8,46 – 158,5	7,01 – 10,71 / 131,34 – 191,28	par / par	1 / 3
Th17/ $CD38^+$	128	127 / 115	9,2 – 58,2 %	8,14 – 10,3 / 51,73 – 65,46	1,87 – 37,46	X	par / npar	1 / 13
Th17/ $CD38^+$ $KI67^+$	128	111 / 109	<0,2 %	X	<0,11	X	npar / npar	17 / 19

$CD8^+$ /$CD38^+$	128	127 / 127	6,35 – 32,49 %	5,72 – 11,79 / 29,28 – 36,04	10,7 – 161,16	8,89 – 12,83 / 136,18 – 190,69	par / par	1 / 1
$CD8^+$ /$Ki67^+$ $CD38^+$	128	119 / 119	<0,61 %	0,54 – 0,62	<2,37	1,51 – 2,23	npar / npar	9 / 9
DN	128	128 / 123	1,3 – 19,1 %	1,08 – 1,53 / 16,1 – 22,65	15,23 – 235,42	12,76 – 18,19 / 197,22 – 281,01	par / par	none / 5
DN/$CD38^+$	128	125 / 127	3,8 – 31,5 %	3,23 – 4,89 / 26,91 – 32,35	1,39 – 31,16	1,03 – 1,82 / 26,26 – 36,94	npar / par	3 / 1
DN/$CD38^+$ $KI67^+$	128	119 / 116	<0,6 %	0,47 – 0,73	<0,34	X	npar / npar	9 / 12
DP	128	126 / 127	0,2 – 2,9 %	0,14 - 0,25 / 2,41 – 3,46	1,67 – 46,64	1,35 – 2,07 / 37,75 – 57,63	par / par	2 / 1
DP/$CD38^+$	128	128 / 127	6,9 – 63,3 %	5,62 – 8,41 / 51,86 – 77,61	<10,66	8,77 – 18,95	par / npar	none / 1
DP/$CD38^+$ $KI67^+$	128	126 / 111	<6,3 %	5,45 - 12,4	<0,25	X	npar / npar	2 / 17

3.2 T cell panel 2

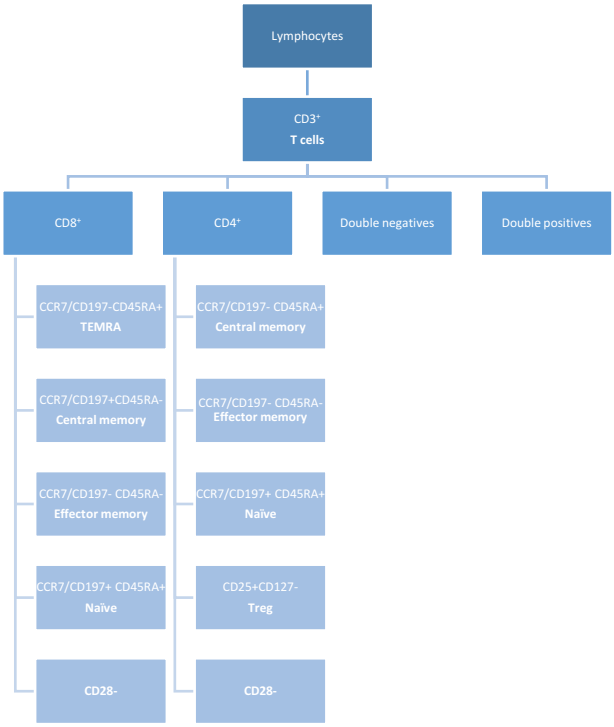
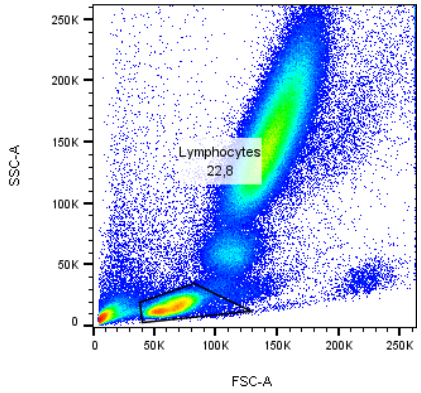


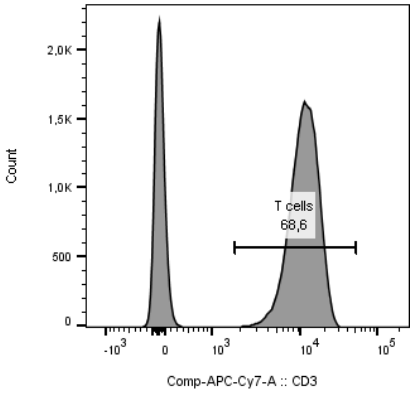
Figure 7 Gating strategy for the T2 panel

Similar to panel 1, the lymphocytes and subsequently T cells were gated, which were then gated for CD4 and CD8. The $CD4^+$ cells were subcategorized into effector memory cells ($CD45RA^-$, $CCR7^-$), central memory cells ($CD45RA^-$, $CCR7^+$), naïve cells ($CD45RA^+$, $CCR7^+$) and regulatory T cells ($CD25^+$, $CD127^-$). The $CD8^+$ cells were divided into effector memory ($CD45RA^-$, $CCR7^-$), central memory ($CD45RA^-$, $CCR7^+$), naïve cells ($CD45RA^+$, $CCR7^+$) and TEMRA cells ($CD45RA^+$, $CCR7^-$). Both $CD4^+$ and $CD8^+$ were additionally gated for CD28. Figure 7 and 8 summarize the gating strategy.

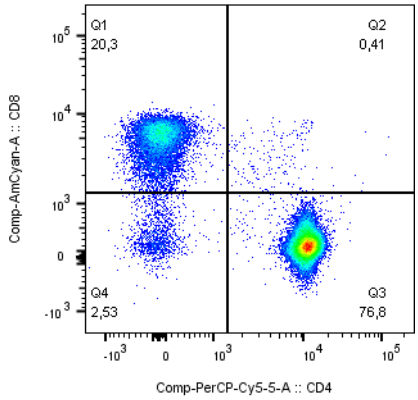
A


Lymphocytes
22,8
SSC-A
FSC-A

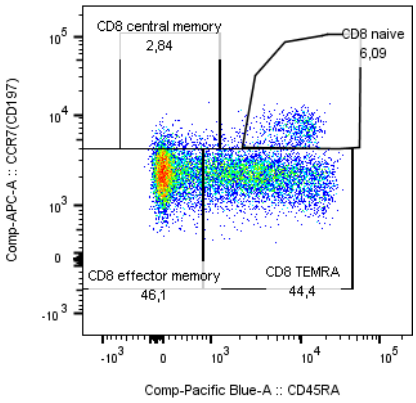
B


T cells
68,6
Count
Comp-APC-Cy7-A :: CD3

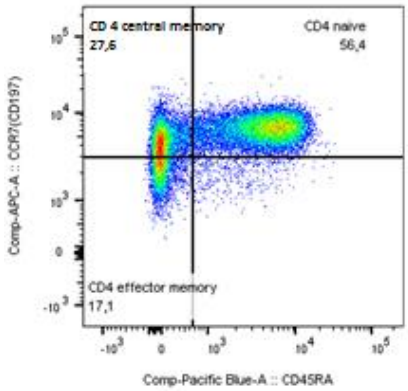
C


Q1
20,3
Q2
0,41
Q4
2,53
Q3
76,8
Comp-AmCyan-A :: CD8
Comp-PerCP-Cy5-5-A :: CD4

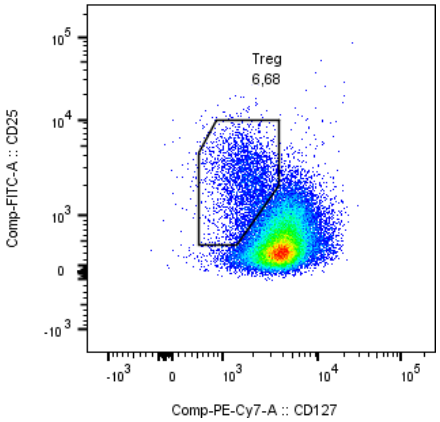
D


CD8 central memory
2,84
CD8 naive
6,09
CD8 effector memory
46,1
CD8 TEMRA
44,4
Comp-APC-A :: CCR7(CD197)
Comp-Pacific Blue-A :: CD45RA

E


CD 4 central memory
27,6
CD4 naive
56,4
CD4 effector memory
17,1
Comp-APC-A :: CCR7(CD197)
Comp-Pacific Blue-A :: CD45RA

F


Treg
6,68
Comp-FITC-A :: CD25
Comp-PE-Cy7-A :: CD127

G

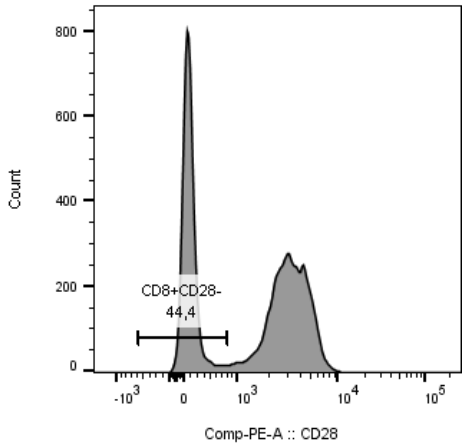


Figure 8 Representative dot plots of the T2 panel gating strategy
A: Determination of lymphocytes based on cell size (FSC) and cell granularity (SSC)
B: Gating of T cells via anti-CD3 antibody staining
C: T cell subclass gating using anti-CD4 and anti-CD8 antibodies
D & E: Subdivison of CD4 and CD8 subsets
F: Gating of regulatory T cells with CD25 and CD127
G: Gating of CD4 and CD8 cells for $CD28^-$ cells

3.2.1 T cell panel 2 reference intervals

Table 11 provides a summary of the T cell panel 2 reference intervals. The numbers were rounded to zero decimal place, if the width of the confidence intervals still contained the reference range. The table shows the total sample size, the sample size after outlier elimination, the percentage (%) and absolute (abs) RI, the method of calculation (parametric (par) or non-parametric (npar)), the 90% confidence intervals for the lower and upper limits of the reference range and the number of outliers. If the population was not normally distributed and the sample size was below 119, the calculation of the CIs was mathematically not possible and was therefore marked with an X.

Table 11 T cell panel 2 reference intervals

Subset	**n** (total)	**n** (%/abs)	**RI** (%)	**90%-CI** (%)	**RI** (abs)	**CI** (abs)	**Parametric / Non-parametric** (%/abs)	**Outlier** (%/abs)
$CD8^+$ TEMRA	128	125 / 125	8,6 – 82,2 %	7,45 – 9,95 / 71,09 – 94,94	17,5 – 388,09	14,65 – 21,74 / 318,53 – 472,84	par / par	3 / 3

$CD8^+$ CM	128	120 / 121	1,1 – 17,5 %	1,04 – 1,47 / 14,7 – 20,77	3,33 – 63,83	2,74 – 4,03 / 52,65 – 77,39	par / par	8 / 7
$CD8^+$ EM	128	122 / 121	12,1 – 71,8 %	10,79 – 13,6 / 64,00 – 80,64	30,87 – 259,65	26,87 – 35,47 / 225,99 – 298,33	par / par	6 / 7
$CD8^+$ naïve	128	123 / 126	5,9-60,5 %	4,67 – 7,07 / 57,54 – 64,56	12,74 – 309,59	8,91 - 16,98 / 269,15 – 354,81	npar / npar	5 / 2
$CD8^+CD28^-$	128	128 / 128	4,3-68,6 %	3,98 – 5,53 / 63,09 – 69,18	10.37 – 447,58	8,17 – 13,17 / 352,57 – 568,2	npar / par	none / none
$CD4^+$ CM	128	121 / 126	11 – 35,3 %	10,14 – 11,82 / 32,69 – 38,08	63,23 – 412,71	56,1 – 71,23 / 366,1 – 465,26	par / par	7 / 2
$CD4^+$ EM	128	122 / 122	13,9 – 61,7 %	12,57 – 15,26 / 55,98 – 67,96	103,76 – 531,10	94,27 – 116,41 / 478,81 – 591,32	par / par	6 / 6
$CD4^+$ naive	128	125 / 126	18,9 – 73 %	17,78 – 20,41 / 64,56 – 79,43	106, 90 – 1000,93	92,66 – 132,22 / 867,64 – 1154,71	npar / par	3 / 2
$CD4^+CD28^-$	128	128 / 128	<15,1 %	10,47 – 25,70	<142,2	123 - 346,73	npar / npar	none / none
Treg	128	126 / 124	6,4 – 20,3 %	5,98 – 6,93 / 18,87 – 21,86	42,45 – 197,75	38,44 – 46,87 / 179,09 – 218,35	par / par	2 / 4
DN T2	128	125 / 127	1,3 – 18,9 %	1,11 – 1,56 / 15,94 – 22,43	12,44 – 295,26	10,17 – 15,22 / 241,37 – 361,18	par / par	3 / 1
DP T2	128	127 /126	0,17 – 2,14 %	0,14 – 0,2 / 1,82 – 2,51	1,80 – 31,92	1,5 – 2,16 / 26,56 – 38,35	par / par	1 / 2

3.3 B cell panel

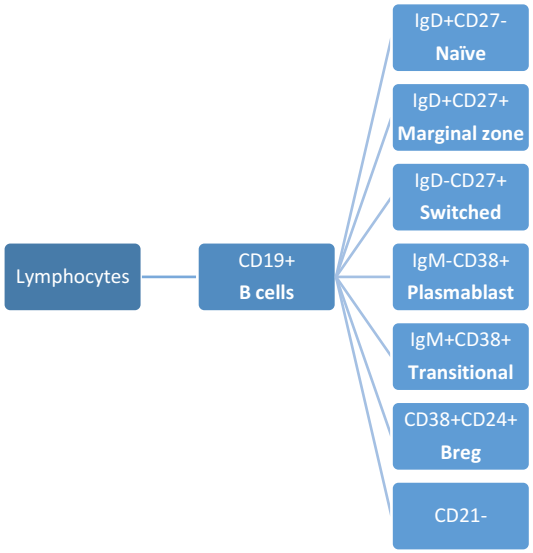
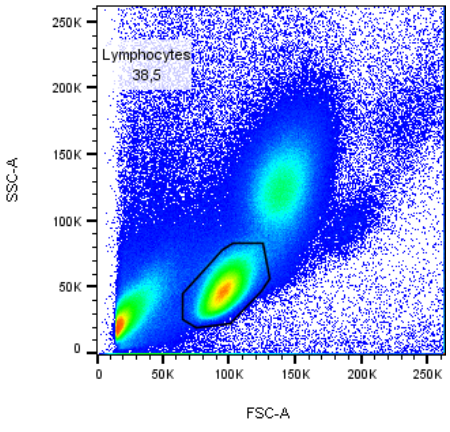


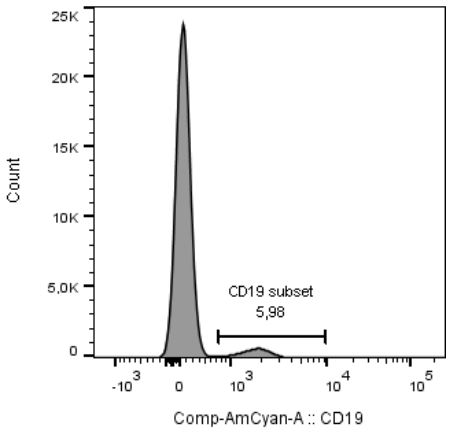
Figure 9 Gating strategy for the B cell panel

After the gating of lymphocytes, CD19 was used as a marker to identify B cells. The B-lymphocytes were then subsequently subcategorized as naïve (IgD^+, $CD27^-$), marginal zone (IgD^+, $CD27^+$), switched (IgD^-, $CD27^+$), transitional (IgM^+, $CD38^+$), plasmablasts (IgM^-, $CD38^+$), regulatory B cells ($CD38^+$, $CD24^+$) and CD21 negative B cells ($CD21^-$, $CD38^-$). Figure 9 and 10 summarize the gating strategy.

A



B



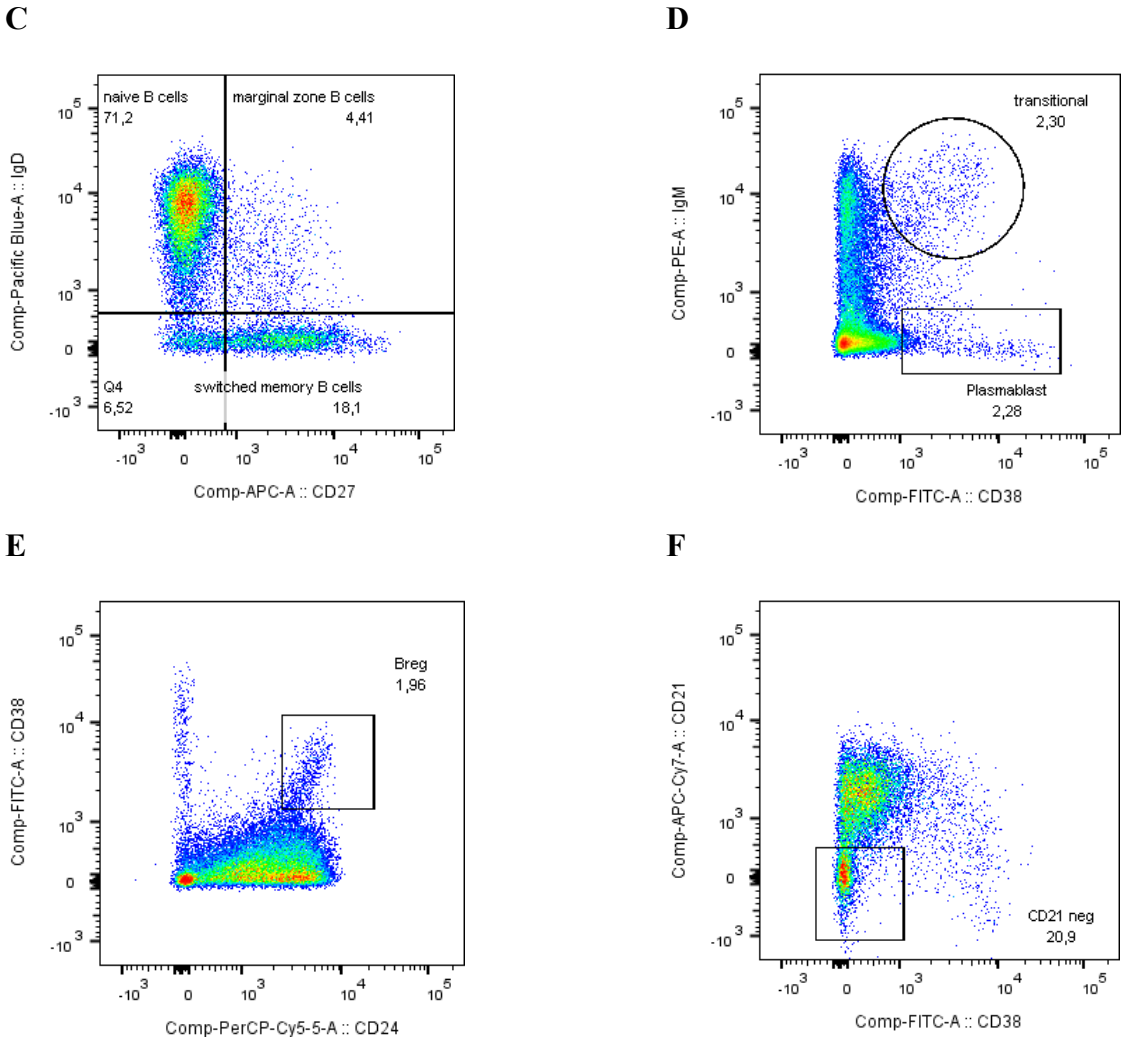


Figure 10 Representative dot plots of the B cell panel gating strategy
A: Determination of lymphocytes based on cell size (FSC) and cell granularity (SSC)
B: Gating of B cells via anti-CD19 antibody staining
C - F: Gating of various B cell subsets

3.3.1 B cell panel reference intervals

Tables 12 provides a summary of the B cell panel reference intervals. The numbers were rounded to zero decimal place, if the width of the confidence intervals still contained the reference range. The table shows the total sample size, the sample size after outlier elimination, the percentage (%) and absolute (abs) RI, the method of calculation (parametric (par) or non-parametric (npar)), the 90% confidence intervals for the lower and upper limits of the reference range and the number of outliers. If the population was not normally distributed and the sample size was below 119, the calculation of the CIs was mathematically not possible and was therefore marked with an X.

Table 12 B cell panel reference intervals

Subset	**n** (total)	**n** (%/abs)	**RI** (%)	**90%-CI** (%)	**RI** (abs)	**CI** (abs)	**Parametric / non-parametric** (%/abs)	**Outlier** (%/abs)
B cells ($CD19^{+}$)	128	123 / 127	2 - 10	1,6 – 2,2 / 9 - 11	77 - 467	60 – 91 / 371 - 575	npar / npar	5 / 1
Bregs	128	127 /126	1,4-13,7 %	1,28 – 16,2 / 9,54 – 18,19	2,12 – 37,54	1,76 – 2,54 / 31,24 – 45,1	npar / par	1 / 2
$CD21^{-}$	128	128 /128	4,6 - 29,8 %	4,08 – 5,17 / 26,49 – 33,53	6,64 – 73,26	5,70 – 7,73 / 62,91 – 85,3	par / par	none / none
Marginal zone B cells	128	128 / 128	5,1-40,1 %	4,47 – 5,81 / 35,19 – 45,68	7,21 – 99,62	6,1 – 8,51 / 84,34 – 117,66	par / par	none / none
Naïve B cells	128	126 / 127	28,4-83,7 %	21,87 – 30,19 / 79,43 – 87,1	34,11 – 336,37	29,48 – 39,45 / 290 – 389,1	npar / par	2 / 1
Plasmablasts	128	126 /127	0,7-4 %	0,69 – 0,703 / 3,98 – 4,01	0,89 – 9,73	0,74 – 1,13 / 9,12 – 10,71	par / npar	2 / 1
Switched memory B cells	128	128 / 127	7-45 %	6,97 – 7,02 / 44,83 – 45,16	10,42 – 105,11	10,36 – 10,47 / 104,51 – 105,72	par / par	none / 1
Transitional B cells	128	124 /126	0,8-6 %	0,67 – 0,94 / 5,07 – 7,09	0,76 – 23,11	0,61 – 0,95 / 18,59 – 28,73	par / par	4 / 2

3.4 *B cell stimulation*

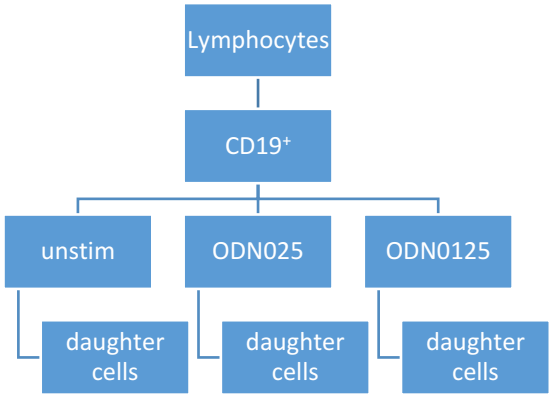
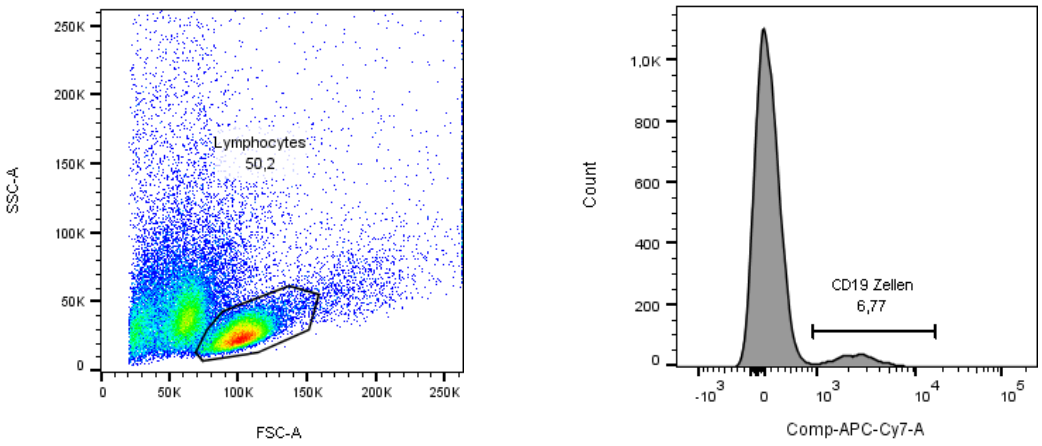


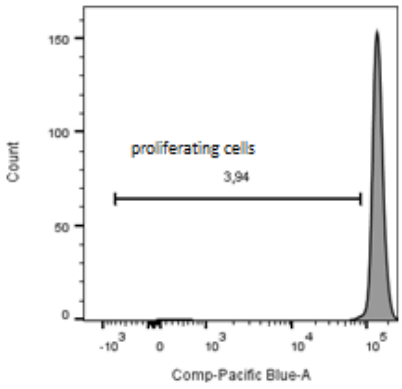
Figure 11 Gating strategy for B cell stimulation

After the gating of the lymphocytes population and the CD19^{+} B cells, CellTrace™ Violet was used to track the number of proliferated cells within the ODN025 and ODN0125 sample, with the unstimulated sample as a control group. Figure 11 and 12 summarize the gating strategy.

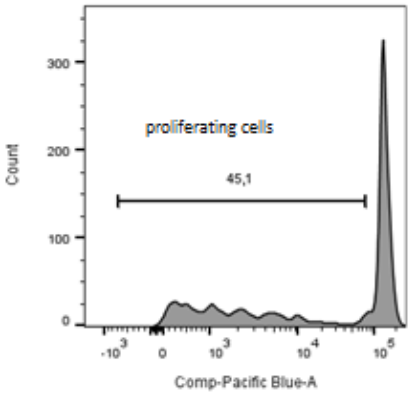
A **B**



C



D



E

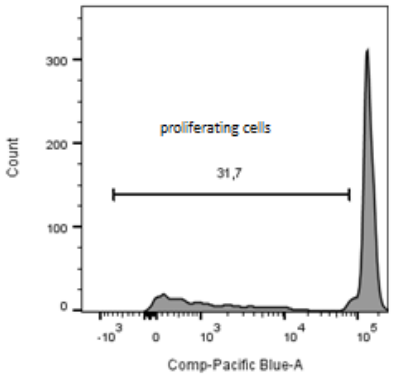


Figure 12 Representative dot plots of the B cell stimulation gating strategy
A & B: Determination of lymphocytes and isolation of T cells
C: Gating of unstimulated proliferating cells
D: Gating of ODN025 stimulated proliferating cells
E: Gating of ODN0125 stimulated proliferating cells

3.4.1 B cell stimulation reference intervals

Table 13 provides a summary of the B cell stimulation reference intervals. The numbers of the RI were only rounded, if the width of the confidence intervals still contained the reference range. The table shows the total sample size, the sample size after outlier elimination, the percentage (%) RI, the method of calculation (parametric (par) or non-parametric (npar)), the 90% confidence intervals for the lower and upper limits of the reference range and the amount of outliers. If the population was not normally distributed and the sample size was below 119, the calculation of the CIs was mathematically not possible and was therefore marked with an X. Furthermore, there are no absolute counts and RIs for the stimulated B (again marked with X).

Table 13 **B cell stimulation reference intervals**

Subset	**n** (total)	**n** (after elimination)	**RI** (%)	**90%-CI** (%)	**Parametric / non-parametric**	**Outlier**
B cells unstim proliferating cells	119	116	3,1-35 %	2,67-3,68 / 29,8-41,10	par	3
B cells ODN025 proliferating cells	119	117	10-76,2 %	-1,72-21,72 / 64,47-87,92	par	2
B cells ODN0125 proliferating cells	119	116	9,2-71,8 %	X	npar	3

3.5 T cell stimulation

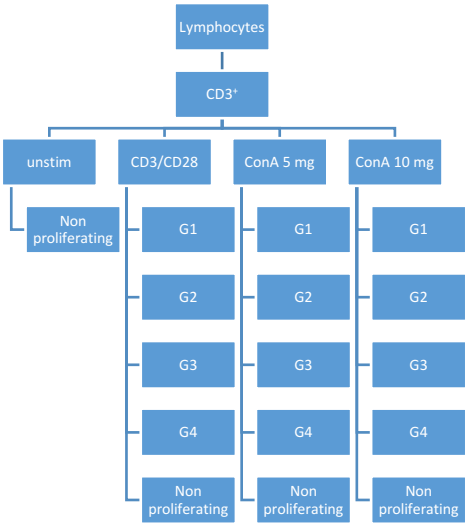
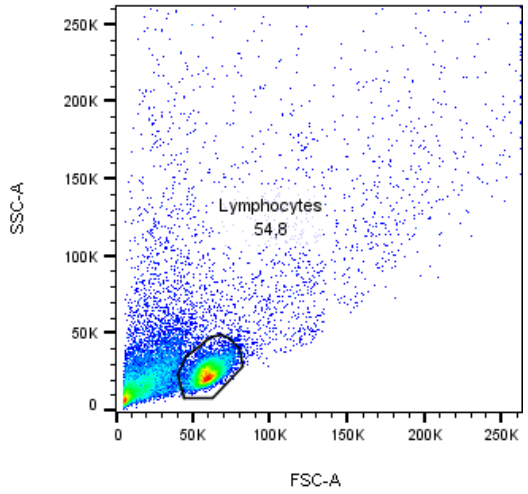


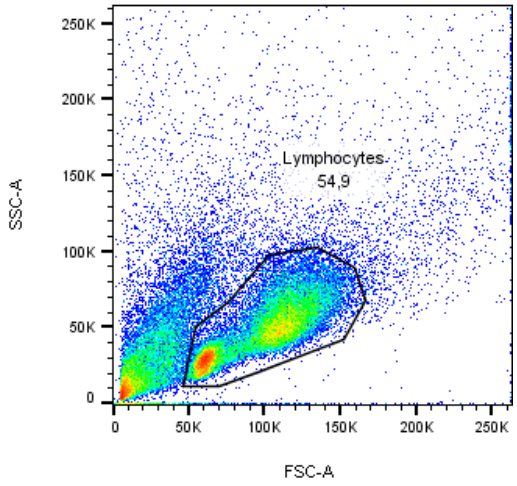
Figure 13 **Gating strategy for T cell stimulation**

After the gating of the lymphocytes and the determination of T cells, the amount of non-proliferating cells within the unstimulated sample were counted as a control group. Then we measured the proliferating cells in the CD3/CD28, ConA 5mg and ConA 10 mg for up to 4 generations as well as the amount of non-proliferating cells. Figure 13 and Figure 14 summarize the gating strategy.

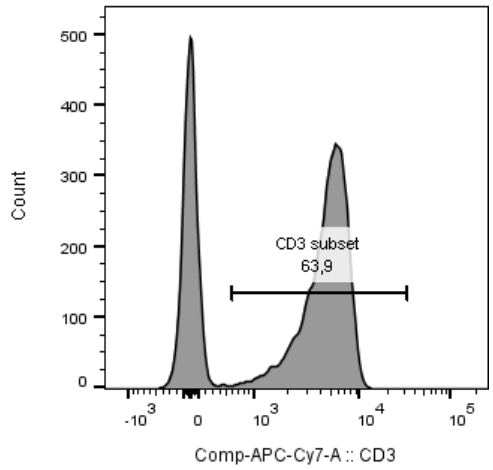
A


Lymphocytes
54,8
SSC-A
FSC-A

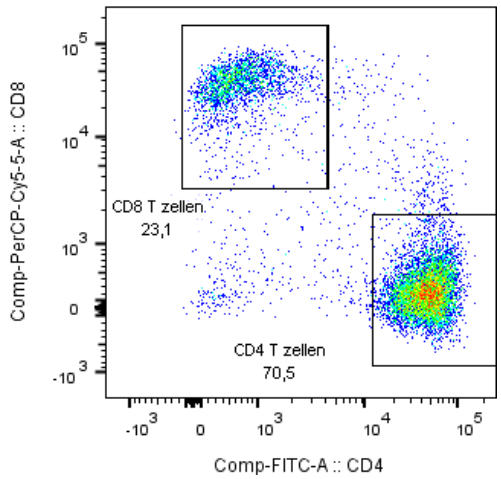
B


Lymphocytes
54,9
SSC-A
FSC-A

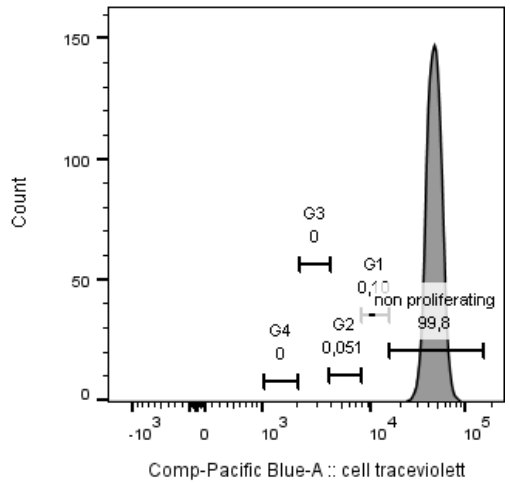
C


CD3 subset
63,9
Count
Comp-APC-Cy7-A :: CD3

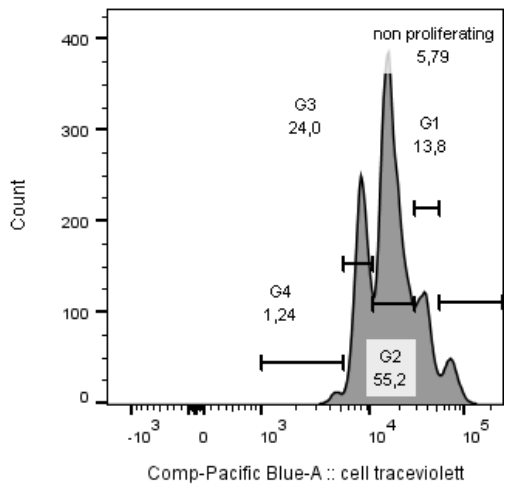
D


CD8 T zellen
23,1
CD4 T zellen
70,5
Comp-PerCP-Cy5-5-A :: CD8
Comp-FITC-A :: CD4

E


G3
0
G1
0,10
non proliferating
99,8
G4
0
G2
0,051
Count
Comp-Pacific Blue-A :: cell traceviolett

F


non proliferating
5,79
G3
24,0
G1
13,8
G4
1,24
G2
55,2
Count
Comp-Pacific Blue-A :: cell traceviolett

G **H**

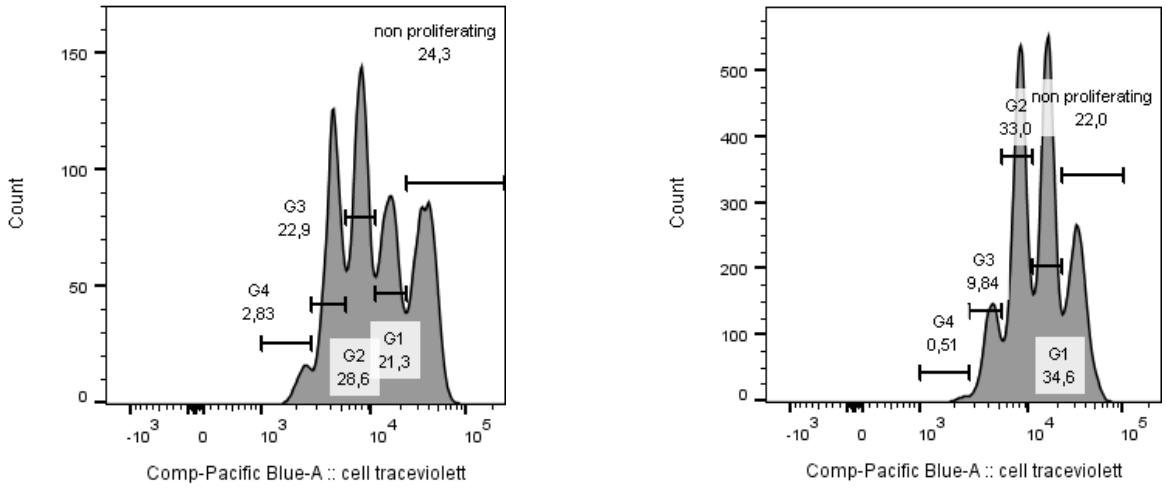


Figure 14 Representative dot plots of the T cell stimulation gating strategy
A: Lymphocyte gating from unstimulated population
B: Lymphocyte gating from stimulated population
C & D: Determination of lymphocytes and isolation of T cells and subsequently $CD4^+$ and $CD8^+$ cells
E: Gating for proliferated generation of unstimulated T cells
F: Gating for proliferated generation of T cells stimulated with CD3/CD28
G: Gating for proliferated generation of T cells stimulated with ConA 5 mg
H: Gating for proliferated generation of T cells stimulated with ConA 10 mg

3.5.1 T cell stimulation reference intervals

Table 14 provides a summary of the T cell stimulation reference intervals. The numbers of the RI were only rounded, if the width of the confidence intervals still contained the reference range. The table shows the total sample size, the sample size after outlier elimination, the percentage (%) RI, the 90% confidence intervals for the lower and upper limits of the reference range, the method of calculation (parametric (par) or non-parametric (npar)), and the number of outliers. If the population was not normally distributed and the sample size was below 119, the calculation of the CIs was mathematically not possible and was therefore marked with an X. There are no absolute counts and RIs for the stimulated T cells (again marked with X).

In order to improve legibility, the T cell stimulation results are displayed in three separate tables, one for each stimulant.

3.5.1.1 CD3/CD28 stimulation

Table 14 Reference ranges for T cell stimulation with CD3/CD28

Subset	**n** (total)	**n** (after elimination)	**RI** (%)	**90%-CI** (%)	**Parametric / non-parametric**	**Outlier**
CD3CD28/CD4+/ G1	114	111	6,0 – 59,2 %	X	npar	3
CD3CD28/CD4+/ G2	114	104	17,3 – 60,3 %	X	npar	10
CD3CD28/CD4+/ G3	114	109	0,6 - 43,7 %	X	npar	5
CD3CD28/CD4+/ G4	114	104	<6,9 %	X	npar	10
CD3CD28/CD4+/ non-proliferating	114	110	2,8 – 43,4 %	X	npar	4
CD3CD28/CD8+/ G1	114	111	6,3 – 52,2 %	5,37 – 7,32 / 45,36 – 59,98	par	3
CD3CD28/CD8+/ G2	114	106	10,9 – 56,5 %	X	npar	8
CD3CD28/CD8+/ G3	114	99	7,9 – 46,4 %	X	npar	15
CD3CD28/CD8+/ G4	114	114	<22,0 %	X	npar	none
CD3CD28/CD8+/ non-proliferating	114	114	2,1 – 81,5 %	1,51 – 2,9 / 65,25 – 101,81	par	none

3.5.1.2 ConA 5 stimulation

Table 15 Reference ranges for T cell stimulation with ConA 5

Subset	**n** (total)	**n** (after elimination)	**RI** (%)	**90%-CI** (%)	**Parametric / non-parametric**	**Outlier**
ConA5/CD4+/ G1	114	111	8,2 – 50 %	X	npar	3

ConA5/CD4$^+$/ G2	114	111	1,7 – 34,2 %	X	npar	3
ConA5/CD4$^+$/ G3	114	114	<14,7	X	npar	None
ConA5/CD4$^+$/ G4	114	111	<1,4 %	X	npar	3
ConA5/CD4$^+$/ non-proliferating	114	110	23,9 – 92,5 %	X	npar	4
ConA5/CD8$^+$/ G1	114	112	3,8 – 70,7 %	X	npar	2
ConA5/CD8$^+$/ G2	114	107	1,3 – 43,9 %	X	npar	7
ConA5/CD8$^+$/ G3	114	114	<37,9 %	X	npar	none
ConA5/CD8$^+$/ G4	114	112	<16,3 %	X	npar	2
ConA5/CD8$^+$/ non-proliferating	114	114	1,8 – 96,1 %	X	npar	none

3.5.1.3 ConA 10 stimulation

Table 16 Reference ranges for T cell stimulation with ConA 10

Subset	**n** (total)	**n** (after elimination)	**RI** (%)	**90%-CI** (%)	**Parametric / non-parametric**	**Outlier**
ConA10/CD4$^+$/ G1	114	111	5,3 – 38,3 %	X	npar	3
ConA10/CD4$^+$/ G2	114	111	1,3-31,6 %	X	npar	3
ConA10/CD4$^+$/ G3	114	113	<15,5 %	0,76 – 1,13 / 13,55 – 19,96	par	1
ConA10/CD4$^+$/ G4	114	110	<2 %	X	npar	4
ConA10/CD4$^+$/ non-proliferating	114	111	28,8 – 97,3 %	X	npar	3
ConA10/CD8$^+$/ G1	114	112	0,2 – 81, 0 %	X	npar	2

ConA10/CD8$^+$/ G2	114	109	1,5 – 42,5 %	X	npar	5
ConA10/CD8$^+$/ G3	114	114	<31,6 %	X	npar	none
ConA10/CD8$^+$/ G4	114	114	<20,1 %	X	npar	none
ConA10/CD8$^+$/ non-proliferating	114	114	2,0 – 93,0 %	X	npar	none

3.6 Cytokines

Table 15 shows the cytokine's reference intervals. The numbers of the RI were only rounded, if the width of the confidence intervals still contained the reference range. The table provides the total sample size, the sample size after outlier elimination, the reference range, the 90% confidence intervals for the lower and upper limit of the interval, the method of calculation (parametric (par) or non-parametric (npar)) and the number of outliers. In the cases of Interferon α, Interleukin 17A and Interferon γ, Dixon's criteria was used to identify outliers. In case of IL1RA 121 samples showed a level of zero pg/ml, while seven samples showed levels between 13 and 375 pg/ml. These samples were identified as outliers by both our method and Dixon's criteria, resulting in a reference value of zero pg/ml.

Table 17 Cytokine reference intervals

Cytokine	**n** (total)	**n** (after elimination)	**RI** (pg/ml)	**90%-CI**	**Parametric / Non-parametric**	**Outliers**
IL8	128	123	<7,24	7,12 – 8,77	npar	5
IL1RA	128	121	0 – 0	0 – 0	npar	7
TNFalpha	128	124	0,51 – 7,46	0,5 – 1,51 / 6,60 – 11,74	npar	4
IFNalpha	128	128	<0,64	0,38 – 0,86	npar	1
IL18	128	128	<36,56	31,62 – 44,66	npar	none
IL5	128	128	<1,92	1,23 – 5,76	npar	none
IP10	128	123	3,14 – 26,72	2,66 – 3,69 / 23,51 – 30,35	par	5
IL10	128	126	<1,24	1,23 – 1,39	npar	2
Il17A	128	127	<0,29	0 – 0,34	npar	1
IFNgamma	128	127	<1,28	1,13 – 1,75	npar	1

3.7 Correlation

We tested the absolute and relative count of the subsets and the stimulated cells for correlations with the test person's age, CRP levels, BMI and cytokine levels. We did not remove outliers for the calculation of correlations.

3.7.1 Correlation with age

Table 18 shows the correlations with the test person's age, with % marking the relative and # marking the absolute count (cells/µl). For an improved legibility, this table provides only the highly significant correlations (** = p<0,01). The complete table with all significant (* = p<0,05) correlations can be found in Appendix IV.

While the overall B cell count (% and #) as well as the B cell stimulation (%) showed a negative correlation with the factor age, the transitional B cells (#) showed a positive correlation.

We observed a similar trend within the T cell population. The overall count, both relative and absolute, showed a negative correlation with the factor age. T1 and T2 $CD4^+$ cells (%), Th2/$CD38^+$(%), Th2/$Ki67^+CD38^+$ (% and #) and $CD4^+CD28^-$ (% and #) show a positive correlation, while Th1/$CD38^+$, T1 DN (#) and T1 DP/$CD38^+$ (%) and naïve $CD4^+$ (% and #) show negative correlations.

T1 and T2 $CD8^+$ (% and #), T1 $CD8^+/CD38^+$ (#), T2 DN (% and #), and naïve $CD8^+$ show negative correlations with age. However, $CD8^+$ CM (%) and $CD8^+CD28^-$ (%) show a negative correlation.

While the majority of the T cell stimulation shows negative correlations, the non-proliferating CD3CD28-stimulated $CD4^+$ cells as well as the non-proliferating $CD4^+$ cells stimulated with ConA 5 and ConA 10 show a positive correlation with the factor age.

Table 18 Correlation with age

Sample	Correlation coefficient	p
$CD19^+$ B cells #	-,236**	,007
Transitional B cells #	,259**	,003
T1 panel $CD3^+$ %	-,228**	,010

T1 panel $CD4^+$ %	,306**	,000
T1 panel $CD8^+$ %	-,274**	,002
T1 panel $CD8^+$ #	-,388**	,000
T1 panel DN #	-,249**	,005
Th1/$CD38^+$ %	-,257**	,003
Th2/$Ki67^+CD38^+$ %	,244**	,006
Th2/$Ki67^+CD38^+$ #	,228**	,010
T1 panel $CD8^+/CD38^+$ #	-,407**	,000
T1 panel DP/$CD38^+$ %	-,329**	,000
T2 panel $CD8^+$ %	-,284**	,001
T2 panel $CD8^+$ #	-,375**	,000
T2 panel $CD4^+$ %	,322**	,000
T2 panel DN %	-,261**	,003
T2 panel DN #	-,315**	,000
$CD4^+$ naïve %	-,267**	,002
$CD4^+$ naïve #	-,226**	,010
$CD4^+CD28^-$ %	,321**	,000
$CD4^+CD28^-$ #	,305**	,000
$CD8^+$ CM %	,296**	,001
$CD8^+$ naïve %	-,541**	,000
$CD8^+$ naïve #	-,640**	,000
$CD8^+CD28^-$ %	,291**	,001
B stim proliferating cells (unstim) %	-,322**	,000
B stim proliferating cells (ODN025) %	-,316**	,000
B stim proliferating cells (ODN0125) %	-,242**	,008
T-Stim CD3CD28/$CD4^+$/total (%)	,397**	,000
T-Stim CD3CD28/$CD8^+$/total (%)	-,415**	,000
T-Stim ConA5/$CD4^+$/total (%)	,322**	,000
T-Stim ConA5/$CD8^+$/total (%)	-,375**	,000
T-Stim ConA5/$CD4^+$/G1 (%)	-,326**	,000
T-Stim ConA5/$CD4^+$/G2 (%)	-,291**	,002

T-Stim ConA5/CD4^{+}/non-proliferating (%)	,321**	,001
T-Stim ConA10/CD8^{+}/total (%)	-,307**	,001
T-Stim ConA10/CD4^{+}/non-proliferating (%)	,282**	,002
T-Stim ConA10/CD4^{+}/G2 (%)	-,316**	,001

Figure 15 displays some of the above listed correlations as dotplots.

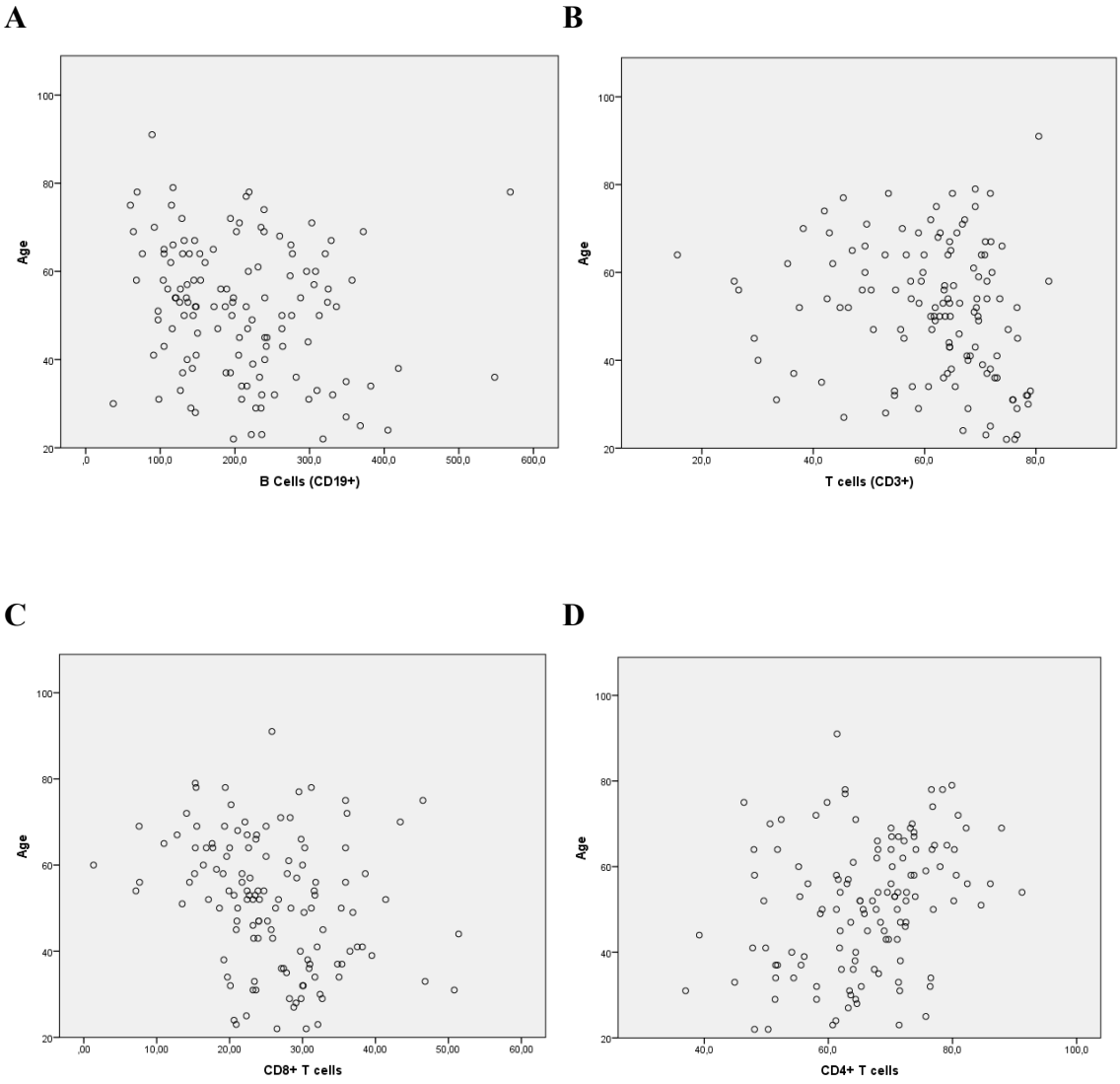


Figure 15 Correlations with age
A: Between age (in years) and B cells (#)
B: Between age (in years) and T cells (%)
C: Between age (in years) and CD8^{+} T cells (%)
D: Between age (in years) and CD4^{+} T cells (%)

3.7.2 Correlation with CRP levels

Table 19 shows the correlations with CRP, with % marking the relative and # marking the absolute count (cells/µl). Furthermore, the correlation coefficient and the level of significance are divided in significant (* = p<0,05) and highly significant (** = p<0,01).

B cell, $CD4^{+}$ and $CD8^{+}$ subsets tend to result in positive correlations with CRP levels, the $CD38^{+}$, i.e. activated cells, Th1 (%) and DP (%) cells show a negative correlation. We also found a positive correlation between CRP and BMI.

Table 19 Correlation with CRP

Sample	Correlation coefficient	p
Th1 cells (%)	,211*	,017
Th1 cells (#)	,211*	,017
Th1/$CD38^{+}$ (%)	-,179*	,043
DP T1 panel (#)	,186*	,036
DP T1 panel/$CD38^{+}$ (%)	-,218*	,013
DN T1 panel (%)	-,178*	,044
$CD4^{+}$CM (#)	,230*	,009
$CD4^{+}$EM (#)	,204*	,021
$CD4^{+}CD28^{-}$ (#)	,180*	,042
CD8 naïve cells (%)	-,193*	,029
Marginal zone B cells (%)	,236**	,007
Marginal zone B cells (#)	,306**	,000
BMI	,290**	,001

Figure 16 displays some of the above listed correlations as dotplots.

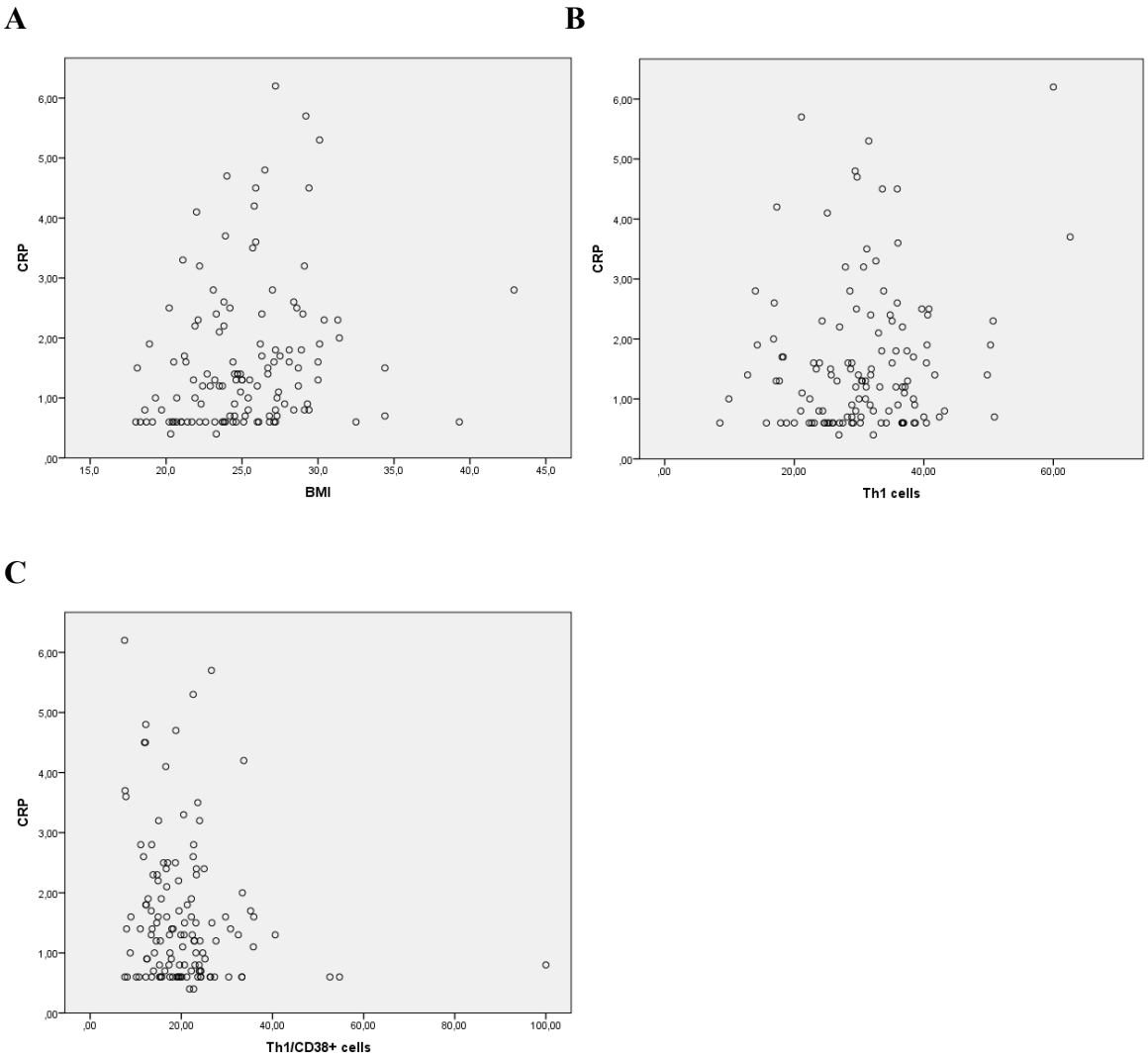


Figure 16 Correlations with CRP
A: Between CRP (mg/dl) and BMI
B: Between CRP (mg/dl) and Th1 cells (%)
C: Between CRP (mg/dl) and Th1/CD38$^+$ cells (%)

3.7.3 Correlations with BMI

Table 20 shows the correlations with the body mass index, with % marking the relative and # marking the absolute count (cells/µl). Furthermore, the correlation coefficient and the level of significance are divided in significant (* = p<0,05) and highly significant (** = p<0,01).

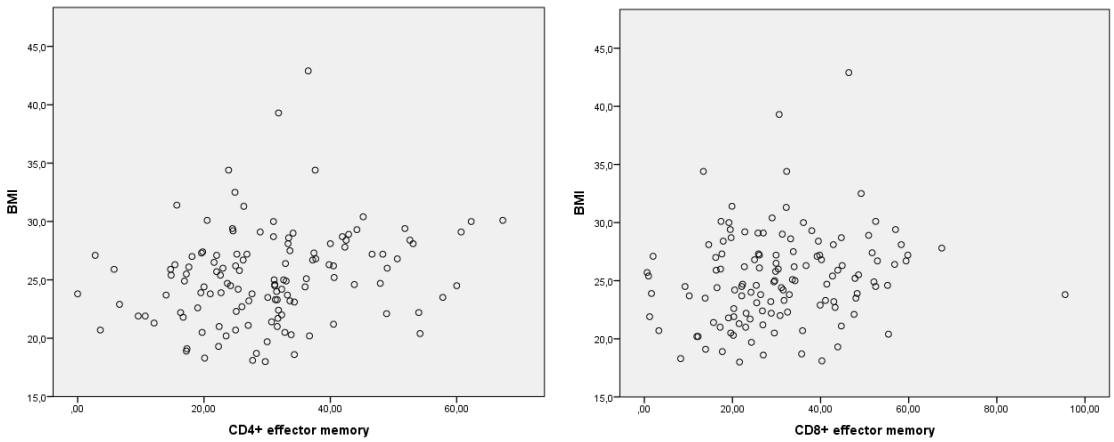
We found negative correlations with BMI within activated (CD38$^+$) Th1_17 (%), Th2 (%) and DP (#) cells as well with CD4$^+$ and CD8$^+$ naïve cells (% and #). CD4$^+$ and CD8$^+$ memory cells and CRP levels showed a positive correlation.

Table 20 Correlation with BMI

Variable	Correlation coefficient	p
Th1_17/CD38$^+$ (%)	-,178*	,044
Th2/CD38$^+$ (%)	-,232**	,009
DP T1 panel (#)	-,214*	,015
DP T1 panel/CD38$^+$ (#)	-,284**	,001
CD4$^+$CM (%)	,259**	,003
CD4$^+$EM (%)	,226*	,010
CD4$^+$EM (#)	,185*	,036
CD4$^+$ naïve (%)	-,255**	,004
CD4$^+$ naïve (#)	-,200*	,024
CD8$^+$EM (%)	,215*	,015
CD8$^+$ naïve (%)	-,305**	,000
CD8$^+$ naïve (#)	-,350**	,000
CRP	,290**	,001

Figure 17 displays some of the above listed correlations as dotplots.

A **B**



C D

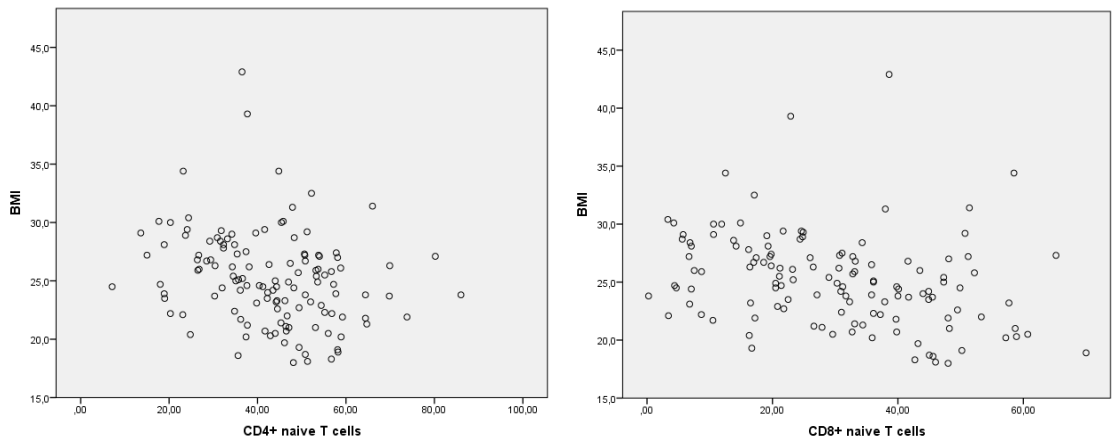


Figure 17 Correlations with BMI
A: Between BMI and CD4$^+$ EM cells (%)
B: Between BMI and CD8$^+$ EM cells (%)
C: Between BMI and CD4$^+$ naive cells (%)
D: Between BMI and CD8$^+$ naive cells (%)

3.7.4 Correlations with cytokine levels

Table 21 shows the correlations with the cytokines, with % marking the relative and # marking the absolute count (cells/µl). For an improved legibility, this table provides only the highly significant correlations (** = p<0,01). The complete table with all significant (* = p<0,05) correlations can be found in Appendix V.

Table 21 Correlation with cytokines

Variables	Correlation coefficient	p
IFNγ with TNFα	,318**	,000
IFNγ with IL5	,579**	,000
IFNγ with IL17A	,692**	,000
IL17A with B stim daughter cells (unstim) (#)	,356**	,000
IL17A with TNFα	,523**	,000
IL17A with IL5	,737**	,000
IL17A with IL10	,433**	,000
IL10 with Th1/Ki67$^+$CD38$^+$ (%)	,292**	,001

IL10 with Th1/Ki67^{+}CD38^{+} (#)	,299**	,001
IL10 with Tregs (%)	,273**	,002
IL10 with CD21^{-} B cells (%)	,226**	,010
IL10 with Plasmablasts (%)	,207**	,019
IL10 with transitional B cells (%)	,302**	,001
IL10 with IL1RA	,250**	,004
IL10 with IL8	,424**	,000
IL10 with IL5	,254**	,004
IL10 with TNFα	-,343**	,000
IL10 with IFNγ	,344**	,000
IL10 with T-Stim CD3CD28/CD8^{+}/NonPro (%)	,264**	,004
IL18 with IL8	,345**	,000
IFNα with IL8	,542**	,000
IFNα with IL1RA	,349**	,000
IFNα with TNFα	,474**	,000
IFNα with T-Stim CD3CD28/CD8^{+}/G1 (%)	,320**	,001
IFNα with T-Stim ConA5/CD4^{+}/G4 (%)	-,274**	,003
IFNα with T-Stim ConA5/CD8^{+}/G1 (%)	,275**	,003
IP-10 with Th2/Ki67^{+}CD38^{+} (%)	,275**	,002
IP-10 with Th2/Ki67^{+}CD38^{+} (#)	,249**	,005
IP-10 with IL5	,291**	,001
IP-10 with T-Stim CD3CD28/CD8^{+}/G3 (%)	-,322**	,000
IP-10 with T-Stim ConA10/CD8^{+}/G3 (%)	-,316**	,001
IL5 with DP T1 panel (%)	,253**	,004
IL5 with DP T1 panel (#)	,235**	,008

IL5 with B stim proliferating cells (unstim) (#)	,280**	,022
IL5 with TNFα	,384**	,000
IL5 with IFNγ	,579**	,000
IL1RA with Tregs	,231**	,009
IL1RA with DP T1 panel/$CD38^+$ (#)	,321**	,000
IL1RA with Bregs (%)	,287**	,001
IL1RA with Bregs (#)	,280**	,001
IL1RA with with transitional B cells (%)	,283**	,001
IL1RA with IL8	,473**	,000
TNFα with Th1/$Ki67^+CD38^+$ (%)	-,242**	,006
TNFα with Th1/$Ki67^+CD38^+$ (#)	-,274**	,002
TNFα with Th2/$Ki67^+CD38^+$ (%)	-,264**	,003
TNFα with DN T1 panel/$CD38^+$ (%)	-,286**	,001
TNFα with T-Stim CD3CD28/$CD4^+$/NonPro (%)	-,246**	,008
TNFα with T-Stim ConA5/$CD4^+$/G1 (%)	,280**	,003
TNFα with T-Stim ConA5/$CD4^+$/NonPro (%)	-,274**	,003
IL8 with Th1/$Ki67^+CD38^+$ (%)	,265**	,002
IL8 with Th1/$Ki67^+CD38^+$ (#)	,300**	,001
IL8 with Tregs (%)	,236**	,007

3.8 Differences regarding gender and smoker status

We investigated the test person's age as well as their B and T cell subsets, the cytokines and the B and T cell stimulation in terms of difference between gender and smoker status.

3.8.1 Gender

Table 21 shows the different subsets with % marking the relative and # marking the absolute count (cells/µl). Furthermore, means by gender, standard deviation and level of significance ($p<0,05$) are listed. The numbers were rounded to zero decimal place.

In 30 out of 153 investigated cases we found a significant difference between the respective arithmetic means separated by gender.
In 18 out of the 30 cases, female test persons showed significantly higher absolute and relative cell counts, for example a 21% higher absolute count of B cells ($CD19^+$) (see Figure and a 14 % higher absolute count of T cells ($CD3^+$) (see Figure . No differences were found within the stimulated B cell population.

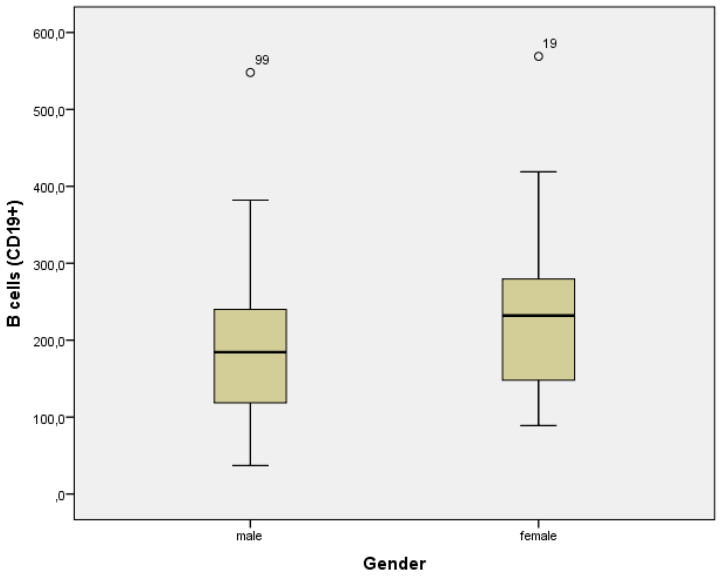
Table 22 Differences between gender

Subset	Mean female (SD)	Mean male (SD)	p
CD19 #	232 (93)	191 (94)	,008
MZ B cells #	40 (27)	28 (20)	,009
Switched B cells #	46 (31)	36 (24)	,038
$CD3^+$ #	1391 (397)	1215 (451)	,006
T1 panel $CD4^+$ #	931 (278)	792 (334)	,002
T2 panel $CD4^+$ #	945 (292)	774 (316)	,001
$CD4^+$ naïve #	445 (225)	320 (185)	,001
DP T1 panel %	1,3 (2,1)	0,8 (0,5)	,016
DP T1 panel #	19 (32)	9 (9)	,000
$DP/CD38^+$ #	4 (4)	3 (5)	,004
$DP/Ki67^+/CD38^+$ %	1 (1)	2,5 (3)	,000
DP T2 panel #	12 (9)	8 (8)	,006
Th1 #	286 (101)	228 (100)	,001
Th1/ $CD38^+$ #	58 (28)	46 (35)	,002
Tregs %	11 (4)	12 (3)	,034
$CD8^+$EM %	27 (15)	34 (15)	,020
$CD8^+$ naïve %	34 (15)	27 (16)	,030
$CD8^+$ naïve #	122 (80)	93 (77)	,022
IL8 #	0,6 (1,6)	2 (3,4)	,006
IL17A #	0,00 (0)	0,12 (0,9)	,044

T Stim CD3/CD28 $CD4^+$ G1 %	33 (12)	24 (13)	,000
T Stim CD3/CD28 $CD4^+$ G3 %	12 (9)	18 (13)	,008
T Stim CD3/CD28 $CD8^+$ G2 %	34 (12)	26 (14)	,001
T Stim CD3/CD28 $CD8^+$ NonPro %	16 (13)	25 (23)	,031
T Stim ConA5 $CD4^+$ G2 %	12 (8)	16 (10)	,023
T Stim ConA5 $CD4^+$ G3 %	3 (3)	4 (4)	,040
T Stim ConA10 $CD4^+$ G3 %	3 (3)	5 (6)	,022
T Stim ConA10 $CD8^+$ G1 %	35 (24)	26 (22)	,041

Figure 18 displays some of the above listed differences as boxplots.

A



B

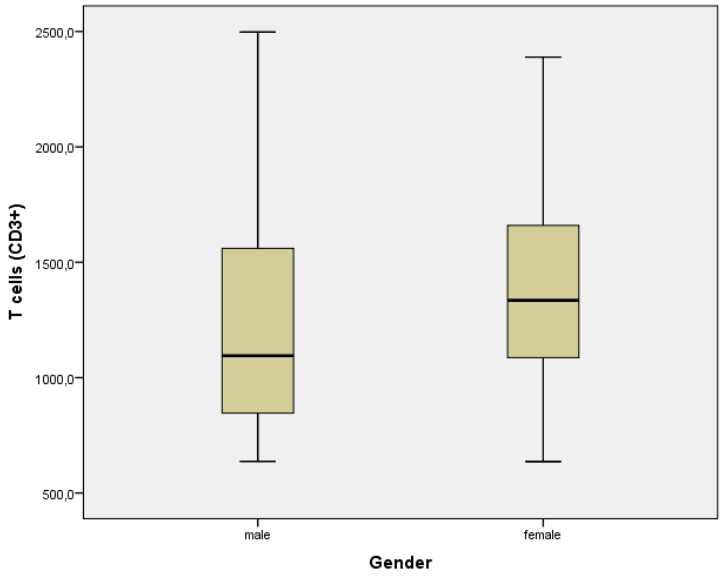


Figure 18 Differences between females and males
A: CD19$^+$ B cells (#)
B: CD3$^+$ T cells (#)

3.8.2 Smoking Status

Table 22 shows the different subsets with % marking the relative and # marking the absolute count (cells/µl). Furthermore, means by smoking status, standard deviation and level of significance ($p<0,05$). The numbers were rounded to zero decimal place.

In 17 out of 153 investigated cases we found a significant difference between the respective arithmetic means separated by smoking status (smoker vs non-smoker). In 13 of the 17 cases, smokers showed significantly higher absolute and relative cell counts. No differences were found within the stimulated B cell population.

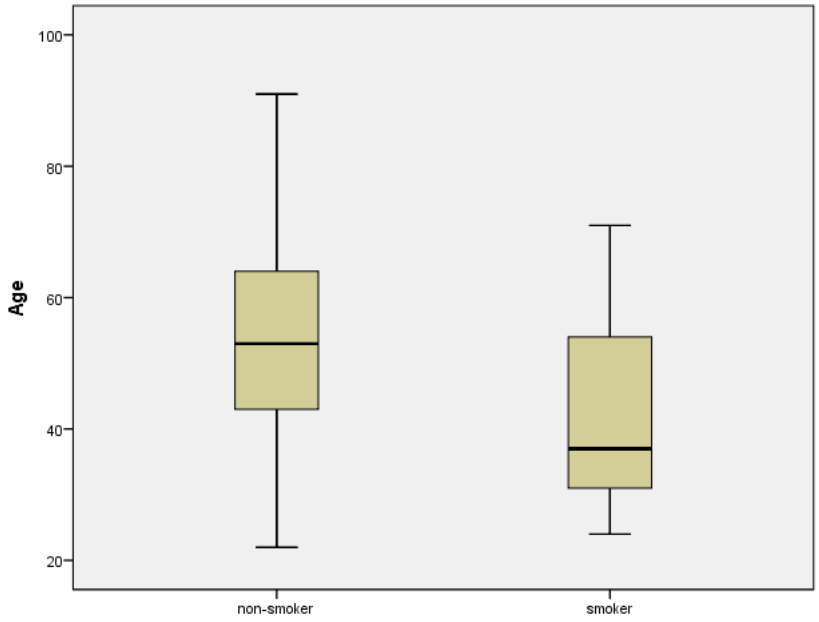
Table 23 Difference between smokers and non-smokers

Subset	Mean smoker (SD)	Mean non-smoker (SD)	p
Age (years)	44 (15)	53 (15)	,007
MZ B cells %	19 (7)	16 (10)	,005
MZ B cells #	43 (24)	31 (24)	,009
Naïve B cells %	50 (14)	61 (15)	,001

Switched B cells %	25 (10)	19 (9)	,003
Switched B cells #	58 (40)	36 (22)	,015
Th2 #	66 (31)	54 (43)	,020
Th2/CD38^{+} #	12 (8)	10 (24)	,020
CD4^{+} CM #	211 (91)	176 (99)	,040
Tregs #	112 (41)	95 (44)	,041
CD8^{+} CM #	27 (17)	24 (37)	,002
CD8^{+}CD28^{-} %	20 (11)	30 (19)	,027
CD8^{+} naïve #	151 (98)	95 (71)	,011
DP T2 panel #	13 (11)	9 (8)	,028
IL5 #	0,00 (0,0)	0,23 (0,73)	,024
T Stim CD3/CD28 CD4^{+} G2 %	45 (13)	37 (15)	,015
T Stim ConA5 CD4^{+} G1 %	29 (9)	25 (15)	,027

Figure 19 displays some of the above listed differences as boxplots.

A



B

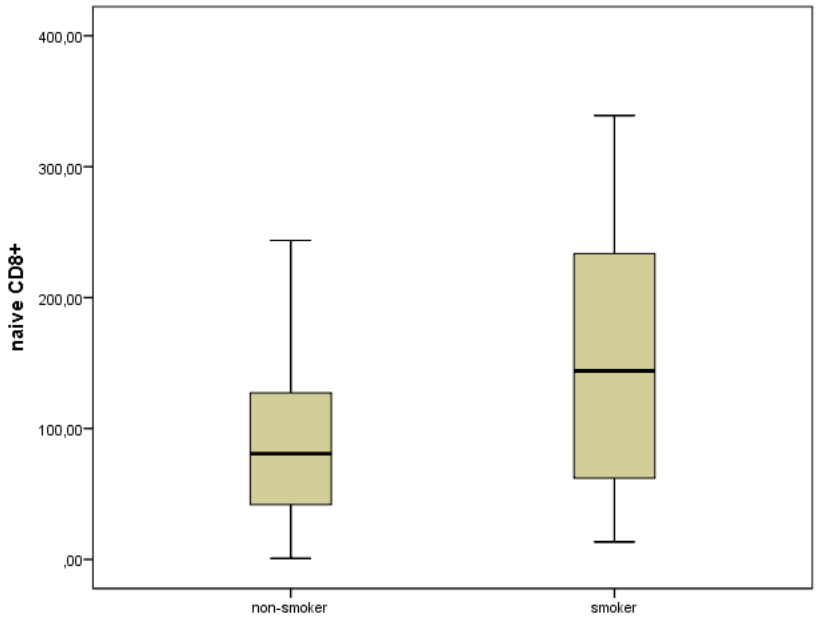


Figure 19 Differences between non-smokers and smokers
A: Age (in years)
B: Naïve $CD8^+$ cells (#)

4 Discussion

The aim of this study was to determine reference intervals for an extended spectrum of lymphocyte subsets in a healthy Austrian population, both to investigate the state of a healthy immune system and to establish reference ranges that are applicable for the Department of Rheumatology and Immunology's clinical laboratory diagnostics. Since the immune system is influenced by a variety of factors, it is essential to compare one's results with similar studies in current scientific literature.

4.1 Comparison of our reference intervals with other studies

There are numerous studies from different countries in which reference ranges have been established. Examples are Al-Mawali et al. (2013) in Oman (173), by Qin et al. (2016) in China (174) or by Valiathan et al. (2014) in the USA (175), but because ethnic background as well as geographical and environmental factors have a significant influence on the immune system, the strongest comparison is found within similar geographical regions.

Bisset et al. (2004) determined reference intervals in a healthy population of 70 blood donors (26 females, 44 males) in Switzerland (176). The reference intervals were calculated in a non-parametric approach and defined as the 2.5^{th} and 97.5^{th} percentile with no outlier detection nor elimination (176). While T cells (CD3), T helper cells (CD4), cytotoxic lymphocytes (CD8) and B cells (CD19) were gated in a similar manner, Bisset et al. investigated different subsets than our study. The only overlaps were activated CD8/CD38 T cells and both $CD4^+$ and $CD8^+$ naïve cells. However, they gated the naïve cells as CD45RA and CD62L.

In a different study, Kverneland et al. (2016) investigated a total of 98 healthy test persons (based in two cohorts of 52 and 46 subjects) with equal gender distribution (177). They recruited their study sample within the hospital staff of the Charité Berlin, representing a European ethnicity (177). A gating strategy similar to our T2 panel was applied for naïve CD4, CD4 CM, CD4 EM, Tregs, naïve CD8, CD8 CM, CD8 EM and CD8 TEMRA. Furthermore, the B cell panel was also gated with a similar strategy. Kverneland et al. gated

their B cell ($CD19^+$) subsets as naïve ($CD27\text{-}IgD^+$), transitional B cells ($CD27^-$, $CD38^{high}$, IgM^+, $CD24^+$), MZ B cells ($CD27^+$, IgD^+), class-switched B cells ($CD27^+$, IgM^+, $CD38^{demi}$), plasmablasts ($CD27^{high}$, $CD38^{high}$, IgD^-, IgM^-) and $CD21^{low}$ B cells ($CD38^{low}$, $CD21^{low}$) while our gating strategy was defined as naïve (IgD^+, $CD27^-$), marginal zone (IgD^+, $CD27^+$), switched (IgD^-, $CD27^+$), transitional (IgM^+, $CD38^+$), plasmablasts (IgM^-, $CD38^+$), regulatory B cells ($CD38^+$, $CD24^+$) and CD21 negative B cells ($CD38^-$, $CD21^-$).

Another study that investigated B cell reference intervals was published by Morbach et al. (2010), in which they defined reference intervals for B cell subsets in healthy children and adults. The 63 adults were recruited from the University of Wurzburg's staff and medical students (178). A similar gating strategy was executed for naïve (IgD^+, $CD27^-$) and switched (IgD^-, $CD27^+$) B cells. However, Morbach et al. defined transitional B cells as CD24+CD38+ plasmablasts as CD24-CD38+ and CD21- as CD21low and CD38low. The determination of the reference intervals was done by calculating the median, the 25^{th} and the 75^{th} percentile (178).

Table 24 summarizes the relative (%) comparison between the studies while Table 25 summarizes the comparison of the absolute cell count. The results of Kverneland et al. and Morbach et al. are in fact the first and third quartile, which is not the recommended method. The RIs are therefore narrower than ours are. The compared studies neither achieved sample size postulated by the IFCC and NCCLS nor performed outlier detection and elimination, which creates a strong susceptibility for the influence of outliers.

Table 24 Comparison of frequency reference intervals

Subset	**Graz** (n=128)	**Bisset et al.** (n=98)	**Kverneland et al.** (Cohort 1 n=52)	**Kverneland et al.** (Cohort 2 n=46)	**Morbach et al.** (19-25 years, n=31)	**Morbach et al.** (26-50 years n=32)
CD3	42 - 79	55 – 84	66 – 76	67 – 77		
CD4	48 - 86	33 – 63	55 – 67	55 – 70		
CD4 naïve	19 – 73	9 – 42	24 – 46	27 – 52		
CD4 CM	11 – 35		33 – 47	35 – 52		
CD4 EM	14 – 62		11 – 19	11 – 20		
CD4 Tregs	6 – 20		7 – 9	4 – 8		
CD8	15 – 45	12 – 39	28 – 39	26 – 37		

CD8 naïve	6 – 61	4 – 19	13 – 43	21 - 46			
CD8 CM	1 – 18		9 – 17	8 - 15			
CD8 EM	12 – 72		26 – 43	23 – 39			
CD8 TEMRA	9 – 82		8 – 42	10 - 29			
CD8/CD38	6 – 32	1 – 7					
CD19	2 – 10		9 – 14	7 - 13	7 – 11		7 – 11
Naïve B cells	28 – 84		40 – 65	47 - 66	66 - 80		58 – 72
MZ B cells	5 – 40		8 – 16	8 - 21			
Switched	7 - 45		10 – 21	11 – 19	7 - 13		9 - 19
Transitional	1 - 6		1 – 3	2 – 4			
Plasmablasts	1 - 4			1 – 2	1 – 2	1 - 2	1 – 2
CD21neg	5 - 30			4 – 10	6 – 13	1 - 3	2 – 5

Table 25 Comparison of absolute reference intervals (cells/µl)

Subset	**Graz** (n=128)	**Bisset et al.** (n=98)	**Kverneland et al.** (Cohort 1 n=52)	**Kverneland et al.** (Cohort 2 n=46)	**Morbach et al.** (19-25 years, n=31)	**Morbach et al.** (26-50 years n=32)
CD3	645 - 2355	536 – 1787	1055 – 1697	1053 – 1678		
CD4	389 - 1653	309 – 1139	637 – 1050	615 - 1049		
CD4 naïve	107 - 1001	84 – 761	153 – 472	212 – 467		
CD4 CM	63 – 413		254 – 442	259 – 459		
CD4 EM	104 – 531		86 – 161	83 – 157		
CD4 Tregs	42 - 198		47 – 96	26 - 63		
CD8	126 – 754	137 – 823	334 – 527	315 – 542		
CD8 naïve	13 - 310	42 – 360	44 – 205	73 – 190		
CD8 CM	3 - 64		38 – 75	30 – 66		
CD8 EM	31 - 260		84 – 226	87 – 176		
CD8 TEMRA	18 - 388		36 – 154	35 – 137		

CD8/CD38	11 - 161	13 – 124				
CD19	77 - 467		149 – 289	122 - 270	133 – 255	169 – 271
Naïve B cells	34 - 336		24 – 69	71 - 162	92 – 199	112 – 169
MZ B cells	7 - 100		5 – 23	14 - 40		
Switched	10 - 105		8 – 21	17 – 38	10 – 31	18 – 40
Transitional	1 – 23		1 – 4	2 – 10		
Plasmablasts	1 - 10		1 – 2	1 – 3	1 – 3	1 – 3
CD21neg	7 – 73		3 – 9	10 – 23	6 – 17	4 – 11

One aim of this study was to establish reference ranges for a large number of B and T cell subsets. The comparison of our reference intervals with other studies' ranges reveals a certain discrepancy, which is, depending on the subset in question, more or less distinctive. The immune system and its composition are influenced by a vast amount of factors, two of them being ethnic background and environmental effects. To minimalize the impact of ethnic and environmental influences, we compared only studies, which were conducted in central and/or Western Europe and therefore represent a European/Caucasian population exposed to similar ethnical and environmental effects. Even then, certain differences remain between the studies. One reason for those discrepancies is the sample size. The NCCLS (166) and the IFCC (169) recommend a number of at least 119 samples to establish reference intervals. Even though Solberg (2004) states, that it is absolutely possible to calculate RIs for smaller sample sizes (169), the reference ranges become more and more susceptible to the influence of outliers. Especially samples in which no outlier detection or elimination has been performed produce reference intervals that are askew (168). One major factor is also the applied method of reference intervals determination. Depending on whether you calculate the mean ± 1.96 × SD, the 2.5^{th} and 97.5^{th} percentile or the 1^{st} and 3^{rd} quartile, the reference ranges are going to be inevitably different and are only comparable to a certain degree.

4.2 Correlations and gender differences

The C-reactive protein is an acute-phase protein and is widely used as a biomarker for (systemic) inflammation processes within the body. In light of this, we found positive correlations between the CRP levels and Th1, CD4 CM, CD4 EM, and MZ B cells accompanied by a negative correlation with CD8 naïve cells. What we observed as counterintuitive was the fact that the activated $CD38^+$ cells (Th1, T1 DP and T1 DN) showed

negative correlations with CRP. Unfortunately, we were not able to find similar results in current scientific literature.

We also observed a highly significant positive correlation with the body mass index. It was shown by multiple studies that there are positive correlations between CRP and BMI (179,180). Research suggests that an elevated BMI means a high amount of fatty tissue which is considered to be hormonally active and leads to a certain proinflammatory status (181,182). Further correlations between the subsets and BMI were positive with memory cells (CD4 CM, CD4 EM, CD8 EM) and negative with naïve CD4 and CD8 cells as well as with activated (CD38) T1 panel cells (Th1/17, Th2, DP). Some studies suggest that obesity impairs the immune system and reduces the number of $CD8^+$ cells, activated cells and the responsiveness to stimulation (183–185). Nieman et al. (1996) were not able to discover a link between obesity and impaired immune competence (186). Further research needs to elucidate the interdependencies between elevated body weight and the immune system.

In our study, we found significant difference between the lymphocyte levels of smokers and non-smokers. Firstly, the average smoker was younger (44 years) than the average non-smoker (53 years), a development which is consistent with the finding of Austria's statistical office (187). Secondly, smokers display higher relative and absolute counts among B and T cell subsets, which could be related to a proinflammatory status in which their immune system is.

Within the age correlations, we were able to confirm a phenomenon that has been described by previous reports. We observed a process of phenotypical and functional alterations in the immune system of the elderly (188). We detected that $CD19^+$ B cells and $CD3^+$ T cells decline with increasing age. This tendency was also observed among the naïve $CD8^+$ cells. The $CD4^+$ cells were somewhat ambivalent, as the relative count of all $CD4^+$ cells showed a positive correlation whilst the naïve $CD4^+$ cells correlated negatively, which is described as an inverse CD4/CD8 ratio (189). Pera et al. (2015) describe the inverse ratio, the drop of naïve cells and high counts of terminally-differentiated T cells (a precursor being the $CD28^-$ cells) as the hallmarks of immunosenescence (189).

Why exactly the immune system undergoes such changes is still partly speculation. There are various leads that suggest an impaired heavy chain gene recombination in B cells which results in a decline of B cells over the years (190,191). Furthermore, a positive correlation between immunosenescence and chronic viral infection with Cytomegalovirus and Epstein-Barr virus leading to T cell depletion was reported by Vescovini et al. (2004) (192,193).

Highly significant positive correlations were observed in the $CD28^-$ subsets among helper and cytotoxic cells, which is consistent with current literature (177). Koch et al. (2016) describe a process, in which T cells lose the ability to express different CDs and receptors as they age, one of them being CD28 (194). Similar to Ferguson et al. (1995) we observed a negative correlation between age and our stimulated $CD8^+$ T cells, which could be a result of an accumulation of $CD28^-$ cells which react inertly to a stimulation (195). Furthermore, our B cell stimulation showed a negative correlation with age, which indicates a decreased B cell response. This is consistent with studies that report a higher susceptibility towards infections among the elderly (174,189–191), which can be linked back to reduced B cell function.

We also investigated the differences between females and males. We observed that females show a higher absolute count of $CD19^+$ B cells, MZ B cells and switched B cells. Similar observations were made in the T cell panels, where higher absolute and relative counts among $CD3^+$ T cells, $CD4^+$ helper cells, naïve helper and cytotoxic cells and Th1 cells were reported. Melzer et al. (2015) describe similar results, even though they found higher counts in Tregs in females, while we observed more Tregs in males (196). Also we were not able to observe a $CD8^+$ dominance in males, as described by Kverneland et al. (2016) (177). Finally, we observed no significant difference between females and males in the response to the T cell stimulation.

Various studies describe a higher resilience to aging in females (197,198). In many species (199,200) and among humans, females tend to live longer than males, which is linked to a higher immunocompetence towards infections and even cancer (197,201). At the same time, they have a higher susceptibility for autoimmune disorders (202,203). There is no definitive answer why this sexual dimorphism exists in the immune system (198). Different susceptibilities towards aging are found not only in the immune system, but also in the heart muscle (204) and in brain volume (205). It seems likely that hormones are partly responsible for these differences. Kyo et al. (1999) reported that estrogen has an activating influence on telomerase, which could lead to a certain anti-aging effect on a cellular level (206).

Nunn et al. (2009) suggest that the dimorphism in aging and immunocompetence is not a physiological question but an evolutionary. They argue that a higher resilience against diseases leads to improved female fitness (197,199).

To elucidate the connection between aging and gender, further studies need to investigate, which other factors next to hormones might influence the immunosenescence.

4.3 Limitations

The results of this study are limited by different factors, the first one being the manual gating of the FACS data, which is up to certain degree based on a subjective assessment of the investigator. In a majority of the cases, a defined gating strategy helps to minimalize the influence of manual gating but in few cases, the populations were not distributed well enough to determine an explicit gating strategy. If the gating would be done by a different investigator, is could lead to slightly different results. To avoid this problem in the future, it would be interesting to conduct a similar study with the application of an automated gating strategy as described by Mair et al. (2015) in their review (207).
Furthermore, immunophenotyping provides only information about the phenotype of a cell but not about the genotype. The actual function of the investigated cells remains unknown, which is not a restriction establishing reference intervals for a healthy population but should be considered in a similar study with infectious or autoimmune patients.

Another limitation is the sample size of our study. In most cases of the T1, T2 and B cell panel the sample size was >120, which allowed a regular determination of the RIs and the CIs. In some cases, a large number of outliers forced the sample size below the threshold of 119. In those cases, our applied method did not allow us to calculate 90% confidence intervals for the reference values. We were therefore not able to provide a range in which the true value lies.
In case of the B and T cell stimulation, we did not reach our requested sample size of 120, which complicated our calculation of reference ranges and their confidence intervals. Because the stimulation panel took 72 hours (T cells) or 7 days in case of the B cells, in some cases we were not able to perform those stimulations because the end of the stimulation would coincide with a holiday, on which no personal was present to perform the analysis. Furthermore, the B and T cell samples were (extremely) skewed. Despite outlier elimination, it remains questionable whether or not the stimulation reference values represent a valid reference interval and could be applied in clinical use.

We found multiple correlations between our subsets, the test person's age, CRP, BMI and cytokine levels. To identify the genuine relationships between those parameters, regression analysis would have been necessary, but was not performed, because the correlations were

not the focus of this study. A further partitioning of the groups would have provided a more detailed observation of the interdependencies between females and males concerning smoking and aging.

4.4 Conclusion

What can be derived from all that? First of all, it explains that, even though there might be discrepancies between the different studies, there is no one, valid reference interval. Quite the contrary, they are all valid within the population from which they have been calculated. Second of all, this leads to the deduction that valid reference intervals cannot be determined by one centralized laboratory but they need to be established within every country or regions of countries and within every hospital department or laboratory that performs advanced immunological research or patient care. As this task consumes scarce resources like time and money, it might not be feasible for small centers or departments to establish reference intervals. A possible solution is describe in a publication by Maecker et al. (2005) where they applied a standardized protocol for various locations with promising results (208,209).

Another aim of this study was to deepen our understanding of the composition of the healthy immune system. We are now able to describe the frequencies and levels of various subsets within healthy Austrian adults and are therefore able to approach the definition of a healthy immune system in an Austrian population. We also observed that age, gender and lifestyle factors like smoking have a significant influence on the immune system's composition, which should be considered every time an immune cell population is being investigated.

A well-known example for the application of immunophenotyping as a diagnostic method is the determination of a diminished $CD4^+$ population in patients with the human immunodeficiency virus (HIV). It has also been used to diagnose and classify other forms of cellular immune defects. To improve the diagnostic validity of immunophenotyping for more diseases, the next step would be to determine the state of an ill immune system.
As we said in the introduction: To understand sickness we need to understand health first. To compare the immune system in different conditions, further studies with a similar design would need to investigate the subsets in patients with bacterial and viral infections or even autoimmune diseases. If significant differences to a healthy immune system exist, it might

be possible to use this knowledge as an additional tool when it comes to diagnosing patients with immunological and autoimmune diseases.

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Appendix I Patient information and consent form

RefWert014 v2 (Version 2 vom 9.9.2014)

PATIENTENINFORMATION UND EINVERSTÄNDNISERKLÄRUNG

TITEL DER UNTERSUCHUNG:

REFERENZWERTETABLIERUNG FÜR DIE ERWEITERTE LYMPHOZYTENTYPISIERUNG[1], LYMPHOZYTENSTIMULATION[2] UND ZYTOKINBESTIMMUNG[3]

Sehr geehrte(r) Frau/Herr**.....**

Wir laden sie ein, an der oben genannten klinischen Studie teilzunehmen. Die Aufklärung erfolgt im ärztlichen Gespräch.

Ziel der Studie: Die verfeinerte Bestimmung der Untergruppen (Lymphozyten) der weißen Blutkörperchen und ihrer Aktivität ist eine neue Methode die zur Erkennung von Patienten mit Immunmangelerkrankungen beiträgt. Um diese Methode auch in Graz anbieten zu können müsssen zuvor Gesunde und Patienten mit Beeinträchtigungen des Immunsystems getestet werden. Ziel der Studie ist es Referenzwerte für diese neuen Untersuchungen aufzustellen.

Vorgehensweise: Wir bitten sie, uns 45 Milliliter (das entspricht 4 Esslöffeln) Blut für diese Studie zur Verfügung zu stellen.

Risiken und Nebenwirkungen: Wie bei jeder Blutabnahme besteht neben den Schmerzen durch das Einstechen der Nadel das geringe Risiko einer Blutung mit nachfolgender Ausbildung eines blauen Flecks (Hämatom) oder einer Infektion an der Einstichstelle. Zur Vermeidung dieser Komplikation werden die gleichen Vorkehrungen wie bei sonstigen Blutabnahme getroffen (Reinigung der Haut, Alkoholtupfer).

Nutzen und Kosten: Mit Hilfe dieser klinischen Studie sollen Referenzwerte für die Bestimmung der Untergruppen (Lymphozyten) der weißen Blutkörperchen und ihrer Aktivität erstellt werden. Durch die Teilnahme an der Studie ergibt sich für die ProbandInnen kein unmittelbarer persönlicher Nutzen. Die Teilnahme an der Studie erfolgt im Rahmen der Verlaufskontrollen. Somit entstehen **keine** Kosten für die ProbandInnen. Eine allfällige Vergütung von Kosten ist daher nicht vorgesehen.

Datenschutz: Beim Umgang mit Ihren Daten werden die Bestimmungen des Datenschutzgesetztes eingehalten. Zu Ihren namentlichen Daten haben nur die Untersucher Zugang. Diese Personen unterliegen der Schweigepflicht. Die Weitergabe von Daten erfolgt ausschließlich zu statistischen Zwecken, und Sie werden darin ausnahmslos nicht genannt.

Freiwilligkeit: Ihre Einwilligung zu dieser Studie erfolgt freiwillig. Sie können Ihre Teilnahme ablehnen, dies hat für Sie keine nachteiligen Folgen.

[1] Lymphozytentypisierung: Die Bestimmung der verschiedenen Untergruppen der Lymphozyten (= ein Typ von weissen Blutkörperchen)
[2] Lymphozytenstimulation: Aktivierung der Lymphozyten im Reagenzglas.
[3] Zytokinbestimmung: Messung von entzündlichen Botenstoffen im Blut.

Einverständniserklärung: Ich habe die Patienteninformation und Einverständniserklärung gelesen und verstanden. Sollten sich noch weitere Fragen ergeben, kann ich mich jederzeit an Herrn Dr. Martin Stradner, Rheumaambulanz Medizinische Universität Graz, Tel. (0316)-385-81794 wenden.

Zu dieser klinischen Prüfung und der Ihnen vorliegenden Patienteninformation und Einverständniserklärung wurde von der zuständigen Ethikkommission der Medizinischen Universität Graz eine befürwortende Stellungnahme abgegeben.

Ich gebe hiermit freiwillig meine Zustimmung zur Blutabnahme. Eine Kopie dieser Einverständniserklärung wurde mir ausgehändigt.

Name des Patienten	Name des verantwortlichen Arztes
Ort, Datum und Unterschrift des Patienten	Ort, Datum und Unterschrift des verantwortlichen Arztes

Appendix II Case report form

Case report form: **Referenzwert Ermittlung** Gesunde Probanden	Patient study number / patient initials ☐☐ ☐☐☐

1. EIN- und AUSSCHLUSSKRITERIEN:

Neoplastische Erkrankung (aktuell od Anamnese)	ja ☐	nein ☐
Akute oder chronische Infektionserkrankung	ja ☐	nein ☐
Bestehende Schwangerschaft	ja ☐	nein ☐
Schwere Anämie (Hb<9 mg/dl)	ja ☐	nein ☐
Autoimmunerkrankung	ja ☐	nein ☐
Organschäden	ja ☐	nein ☐

2. DEMOGRAPHISCHE DATEN

Geburtsdatum	/ / (Tag/Monat/Jahr)
Geschlecht	

3. ANAMNESE

Erkrankungen:
Derzeitige Therapie:
CRP:

4. LABORWERTE

Blut abgenommen? **a. 5xLi-Heparin (8ml) davon eines auf Eis!** **b. 1x EDTA (3ml)** **ad Immunologie Labor,** **Auenbruggerplatz 8 (Keller Dermatologie)** **8036 Graz**	ja ☐	nein ☐

Appendix III Consent by ethical review committee

Ethikkommission
Medizinische Universität Graz

Auenbruggerplatz 2, A-8036 Graz
ethikkommission@medunigraz.at
Tel.: +43 / 316 / 385-13928, Fax: -14348

VOTUM

gültig bis 12.09.2015

EK-Nummer:	26-559 ex 13/14
Studientitel:	Establishing Reference Values for Lymphocyte Subtyping, Stimulation and Cytokines
Prüfer:	Univ.Prof.Dr. Winfried Graninger Medizinische Universität Graz
Sponsor:	Klinische Abteilung für Rheumatologie und Immunologie
Ansprechpartner:	Univ.Prof.Dr. Winfried Graninger, 8036 Graz, Auenbruggerplatz 15
CRO:	-
Antragsteller:	KAGES
Ansprechpartner:	Dr. Martin Stradner

Die o.a. Studie wurde von der Ethikkommission erstmals im 'expedited Review' am 27.08.2014 behandelt. Die Ethikkommission ist zu folgendem Schluss gekommen:

Es besteht kein Einwand gegen die Durchführung der Studie in der vorliegenden Form.

Kommissionsmitglieder, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher gemäß Geschäftsordnung an der Entscheidungsfindung und Abstimmung nicht teilgenommen haben: keine

Zur Beurteilung vorliegende Dokumente:

Dokumente eingegangen am 08.08.2014, begutachtet im 'expedited Review' am 27.08.2014	
✓ Antragsformular ECS	08.08.2014
✓ Originalprotokoll Protokoll RefWert V1 V1	06.08.2014
Informed Consent Form Patienteneinverständnis-RefWert014v1 V1	06.08.2014
✓ Case Report Form RefWert014 CRF SjSV1 V1	06.08.2014
✓ Case Report Form RefWert014 CRF GesundeV1 V1	06.08.2014
✓ Case Report Form RefWert014 CRF InfektV1 V1	06.08.2014
✓ Sonstiges: Ansuchen auf Gebührenbefreiung V1	06.08.2014
Dokumente eingegangen am 10.09.2014, begutachtet im 'expedited Review' am 12.09.2014	
✓ Antragsformular ECS Unterschriftenseiten	08.08.2014
✓ Informed Consent Form 2	09.09.2014

Die Ethikkommission geht - rechtlich unverbindlich - davon aus, dass es sich um keine klinische Prüfung nach AMG bzw. MPG handelt.

Das Votum der Ethikkommission berührt in keiner Weise die alleinige Verantwortung der Prüferin / des Prüfers / der Prüfer für die ordnungsgemäße Durchführung der Studie unter Einhaltung aller einschlägiger gesetzlicher Bestimmungen und Richtlinien.

Weiters machen wir darauf aufmerksam, dass der Kommission unverzüglich zu melden sind:

- Abweichungen vom Protokoll aus Sicherheitsgründen oder Protokolländerungen

- Änderungen, die das Risiko der Teilnehmer/-innen erhöhen oder die Durchführung der Studie

Medizinische Universität Graz, Auenbruggerplatz 2, A-8036 Graz. www.medunigraz.at
Rechtsform: Juristische Person öffentlichen Rechts gem. Universitätsgesetz 2002. Information: Mitteilungsblatt der Universität und www.medunigraz.at. DVR-Nr: 210 9494, UID: ATU 575 111 79. Bankverbindung: Bank Austria Creditanstalt BLZ 12000 Konto-Nr. 500 948 400 04, Raiffeisen Landesbank Steiermark BLZ 38000 Konto-Nr. 46510.

wesentlich beeinflussen

- Mutmaßliche unerwartete schwerwiegende Nebenwirkungen - SUSARs (AMG-Studien ab 1.5.2004) oder schwerwiegende unerwünschte Ereignisse - SAEs (andere Studien)

- Jegliche Information über sonstige Umstände, die die Sicherheit der Teilnehmer/-innen oder die Durchführung der Studie beeinträchtigen können

Dieses Votum gilt für ein Jahr ab dem Datum der Ausstellung. Bei längerer Studiendauer ist rechtzeitig vor Ablauf der Gültigkeit des Votums ein Zwischenbericht vorzulegen (Berichtsformular), um eine etwaige Verlängerung zu erlangen.

Graz, 12. September 2014

Univ.Prof.DI Dr.Josef Haas
Vorsitzender

Univ.Prof.DDr.Hans-Peter Kapfhammer
Stv. Vorsitzender

Achtung: Bitte bei allen das Projekt betreffende Schreiben oder telefonischen Anfragen die EK-Nummer angeben!

Medizinische Universität Graz, Auenbruggerplatz 2, A-8036 Graz. www.medunigraz.at

Appendix IV Supplementary table of complete age correlation

Table 26 Supplementary table of complete age correlation
*** = significant (p<0,05) and ** = highly significant (p<0,01)**

Sample	Correlation coefficient	p
$CD19^+$ B cells %	-,204*	,021
$CD19^+$ B cells #	-,236**	,007
Breg %	,217*	,014
$CD21^-$ B cells %	,175*	,049
Transitional B cells %	,203*	,021
Transitional B cells #	,259**	,003
Switched B cells #	-,194*	,028
Switched B cells #	-,181*	,041
Naïve B cells #	-,182*	,039
T1 panel $CD3^+$ %	-,228**	,010
T1 panel $CD3^+$ #	-,212*	,016
T1 panel $CD4^+$ %	,306**	,000
T1 panel $CD8^+$ %	-,274**	,002
T1 panel $CD8^+$ #	-,388**	,000
T1 panel DN %	-,194*	,028
T1 panel DN #	-,249**	,005
Th1/$CD38^+$ %	-,191*	,030
Th1/$CD38^+$ %	-,257**	,003
Th2/$Ki67^+CD38^+$ %	,244**	,006
Th2/$Ki67^+CD38^+$ #	,228**	,010
T1 panel $CD8^+/CD38^+$ %	-,197*	,026
T1 panel $CD8^+/CD38^+$ #	-,407**	,000
T1 panel DP/$CD38^+$ %	-,329**	,000
T1 panel DP/$CD38^+$ #	-,211*	,017
T1 panel DN/$CD38^+$ #	-,225*	,011
T2 panel $CD8^+$ %	-,284**	,001
T2 panel $CD8^+$ #	-,375**	,000

T2 panel $CD4^+$ %	,322**	,000
T2 panel DN %	-,261**	,003
T2 panel DN #	-,315**	,000
$CD4^+$ EM %	,200*	,023
$CD4^+$ naïve %	-,267**	,002
$CD4^+$ naïve #	-,226**	,010
$CD4^+CD28^-$ %	,321**	,000
$CD4^+CD28^-$ #	,305**	,000
$CD8^+$ TEMRA %	,221*	,012
$CD8^+$ CM %	,296**	,001
$CD8^+$ EM #	-,214*	,016
$CD8^+$ naïve %	-,541**	,000
$CD8^+$ naïve #	-,640**	,000
$CD8^+CD28^-$ %	,291**	,001
B stim daughter cells (unstim) %	-,322**	,000
B stim daughter cells (ODN025) %	-,316**	,000
B stim daughter cells (ODN0125) %	-,242**	,008
T-Stim CD3CD28/$CD4^+$ (%)	,397**	,000
T-Stim CD3CD28/$CD8^+$ (%)	-,415**	,000
T-Stim CD3CD28/$CD8^+$/G2 (%)	-,216*	,021
T-Stim ConA5/$CD4^+$ (%)	,322**	,000
T-Stim ConA5/$CD8^+$ (%)	-,375**	,000
T-Stim ConA5/$CD4^+$/G1 (%)	-,326**	,000
T-Stim ConA5/$CD4^+$/G2 (%)	-,291**	,002

T-Stim ConA5/CD4^{+}/non-proliferating (%)	,321**	,001
T-Stim ConA10/CD8^{+} (%)	-,307**	,001
T-Stim ConA10/CD4^{+}/non-proliferating (%)	,282**	,002
T-Stim ConA10/CD4^{+}/G2 (%)	-,316	,001

Appendix V Supplementary table of complete cytokine correlation

Table 27 Supplementary table of complete cytokine correlation
*** = significant (p<0,05) and ** = highly significant (p<0,01)**

Variables	Correlation coefficient	p
IFNγ with Th2/CD38^{+} (%)	,192*	,030
IFNγ with Tregs (%)	,221*	,012
IFNγ with B stim daughter cells (unstim) (#)	,204*	,026
IFNγ with TNFα	,318**	,000
IFNγ with IL5	,579**	,000
IFNγ with IP-10	,178*	,044
IFNγ with IL17A	,692**	,000
IFNγ with T-Stim CD3CD28/CD4^{+}/G3 (%)	-,214*	,022
IFNγ with T-Stim CD3CD28/CD4^{+}/G4 (%)	-,208*	,026
IL17A with DN T1 panel/CD38^{+} (%)	-,192*	,030
IL17A with DN T1 panel/Ki67^{+}CD38^{+} (%)	-,221*	,012
IL17A with DN T1 panel/Ki67^{+}CD38^{+} (#)	-,201*	,023
IL17A with B stim daughter cells (unstim) (#)	,356**	,000
IL17A with TNFα	,523**	,000
IL17A with IL5	,737**	,000
IL17A with IL10	,433**	,000
IL17A with IL8	,195*	,027
IL10 with Th1/Ki67^{+}CD38^{+} (%)	,292**	,001

IL10 with Th1/Ki67^{+}CD38^{+} (#)	,299**	,001
IL10 with Th2 (%)	-,210*	,017
IL10 with Th2/Ki67^{+}CD38^{+} (%)	,189*	,033
IL10 with Th2/Ki67^{+}CD38^{+} (#)	,182*	,039
IL10 with CD8^{+}/CD38^{+} (%)	,209*	,018
IL10 with CD4^{+}CD28^{-} (%)	,189*	,033
IL10 with CD4^{+}CD28^{-} (#)	,174*	,049
IL10 with Tregs (%)	,273**	,002
IL10 with Tregs (#)	,178*	,044
IL10 with Bregs (%)	,214*	,015
IL10 with CD21^{-} B cells (%)	,226**	,010
IL10 with Plasmablasts (%)	,207**	,019
IL10 with transitional B cells (%)	,302**	,001
IL10 with transitional B cells (#)	,200*	,024
IL10 with IL1RA	,250**	,004
IL10 with IL8	,424**	,000
IL10 with IP-10	,184*	,038
IL10 with IL18	,215*	,015
IL10 with IL5	,254**	,004
IL10 with TNFα	-,343**	,000
IL10 with IFNα	,215*	,015
IL10 with IFNγ	,344**	,000
IL10 with T-Stim CD3CD28/CD4^{+}/NonPro (%)	,203*	,030
with T-Stim CD3CD28/CD8^{+}/G3 (%)	-,207*	,027

IL10 with T-Stim CD3CD28/$CD8^+$/NonPro (%)	,264**	,004
IL10 with T-Stim ConA5/$CD4^+$/G2 (%)	-,213*	,023
IL10 with T-Stim ConA5/$CD4^+$/G3 (%)	-,208*	,026
IL10 with T-Stim ConA5/$CD4^+$/NonPro (%)	,228*	,015
IL10 with T-Stim ConA5/$CD8^+$/G3 (%)	-,234*	,012
IL10 with T-Stim ConA5/$CD8^+$/G4 (%)	-,202*	,031
IL10 with T-Stim ConA10/$CD4^+$/G3 (%)	-,192*	,041
IL18 with Th1 (#)	,187*	,034
IL18 with $CD4^+CD28^-$ (%)	,179*	,044
IL18 with $CD4^+CD28^-$ (#)	,187*	,034
IL18 with Tregs (#)	,184*	,037
IL18 with IL8	,345**	,000
IL18 with T-Stim CD3CD28/$CD4^+$/G4 (%)	,195*	,037
IL18 with T-Stim ConA5/$CD4^+$/G2 (%)	-,198*	,035
IL18 with T-Stim ConA5/$CD4^+$/G3 (%)	-,210*	,020
IL18 with T-Stim ConA10/$CD4^+$/G1 (%)	-,206*	,028
IFNα with Th2/$Ki67^+CD38^+$ (#)	-,175*	,048
IFNα with DN T1 panel/$CD38^+$ (%)	-,180*	0,42

IFNα with DP T1 panel/CD38^{+} (#)	,205*	,020
IFNα with Tregs (#)	,216*	,014
IFNα with CD8^{+} TEMRA (%)	,198*	,025
IFNα with CD8^{+} TEMRA (#)	,200*	,023
IFNα with IL8	,542**	,000
IFNα with IL1RA	,349**	,000
IFNα with TNFα	,474**	,000
IFNα with T-Stim CD3CD28/CD8^{+}/G1 (%)	,320**	,001
IFNα with T-Stim CD3CD28/CD8^{+}/G2 (%)	-,214*	,022
IFNα with T-Stim ConA5/CD4^{+}/G3 (%)	-,207*	,027
IFNα with T-Stim ConA5/CD4^{+}/G4 (%)	-,274**	,003
IFNα with T-Stim ConA5/CD8^{+}/G1 (%)	,275**	,003
IFNα with T-Stim ConA10/CD8^{+}/G1 (%)	,215*	,022
IP-10 with Th2/Ki67^{+}CD38^{+} (%)	,275**	,002
IP-10 with Th2/Ki67^{+}CD38^{+} (#)	,249**	,005
IP-10 with CD4^{+}EM	,203*	,022
IP-10 with MZ B cells (%)	-,220*	,012
IP-10 with transitional B cells (#)	,202*	,022
IP-10 with IL5	,291**	,001
IP-10 with T-Stim CD3CD28/CD8^{+}/G3 (%)	-,322**	,000

IP-10 with T-Stim CD3CD28/CD8^{+}/G4 (%)	-,204*	,030
IP-10 with T-Stim ConA5/CD8^{+}/G4 (%)	-,185*	,048
IP-10 with T-Stim ConA10/CD4^{+}/G2 (%)	-,209*	,025
IP-10 with T-Stim ConA10/CD8^{+}/G3 (%)	-,316**	,001
IP-10 with T-Stim ConA10/CD8^{+}/G4 (%)	-,189*	,043
IP-10 with T-Stim ConA10/CD8^{+}/NonPro (%)	-,231*	,013
IL5 with DP T1 panel (%)	,253**	,004
IL5 with DP T1 panel (#)	,235**	,008
IL5 with B stim daughter cells (unstim) (%)	,223*	,015
IL5 with B stim daughter cells (unstim) (#)	,280**	,022
IL5 with TNFα	,384**	,000
IL5 with IFNγ	,579**	,000
IL5 with T-Stim ConA10/CD8^{+}/G3 (%)	-,217*	,021
IL5 with T-Stim ConA10/CD8^{+}/G4 (%)	-,202*	,031
IL1RA with Th1/CD38^{+} (#)	,174*	,050
IL1RA with Tregs	,231**	,009
IL1RA with DP T1 panel/CD38^{+} (%)	,223*	,012
IL1RA with DP T1 panel/CD38^{+} (#)	,321**	,000
IL1RA with CD4^{+}CD28^{-} (%)	,223*	,011
IL1RA with CD4^{+}CD28^{-} (#)	,186*	,035

IL1RA with $CD8^{+}$TEMRA (%)	,184*	,038
IL1RA with $CD8^{+}$TEMRA (#)	,200*	,024
IL1RA with $CD8^{+}CD28^{-}$ (%)	,188*	,033
IL1RA with $CD8^{+}CD28^{-}$ (#)	,223*	,011
IL1RA with $CD8^{+}$ naïve (%)	-,197*	,026
IL1RA with Bregs (%)	,287**	,001
IL1RA with Bregs (#)	,280**	,001
IL1RA with Plasmablasts (%)	,179*	,044
IL1RA with with transitional B cells (%)	,283**	,001
IL1RA with transitional B cells (#)	,188*	,034
IL1RA with IL8	,473**	,000
IL1RA with TNFα	,177*	,046
IL1RA with T-Stim ConA10/$CD8^{+}$/G1 (%)	,184*	,050
TNFα with Th1/$Ki67^{+}CD38^{+}$ (%)	-,242**	,006
TNFα with Th1/$Ki67^{+}CD38^{+}$ (#)	-,274**	,002
TNFα with Th2 (%)	,187*	,034
TNFα with Th2/$Ki67^{+}CD38^{+}$ (%)	-,264**	,003
TNFα with Th2/$Ki67^{+}CD38^{+}$ (#)	-,223*	,011
TNFα with Tregs (%)	-,203*	,021
TNFα with DN T1 panel/$CD38^{+}$ (%)	-,286**	,001
TNFα with $CD4^{+}CD28^{-}$ (%)	-,178*	,044

TNFα with B stim daughter cells (unstim) (%)	,184*	,046
TNFα with B stim daughter cells (unstim) (#)	,234*	,010
TNFα with IL8	,182*	,040
TNFα with T-Stim CD3CD28/CD4+/NonPro (%)	-,246**	,008
TNFα with T-Stim ConA5/CD4+/G1 (%)	,280**	,003
TNFα with T-Stim ConA5/CD4+/G2 (%)	,211*	,024
TNFα with T-Stim ConA5/CD4+/NonPro (%)	-,274**	,003
TNFα with T-Stim ConA5/CD8+/G3 (%)	,209*	,026
TNFα with T-Stim ConA5/CD8+/NonPro (%)	-,225*	,016
IL8 with Th1/Ki67+CD38+ (%)	,265**	,002
IL8 with Th1/Ki67+CD38+ (#)	,300**	,001
IL8 with Tregs (%)	,236**	,007
IL8 with Tregs (#)	,207*	,019
IL8 with transitional B cells (%)	,200*	,023
IL8 with T-Stim CD3CD28/CD4+/G1 (%)	-,194*	,038
IL8 with T-Stim CD3CD28/CD4+/G4 (%)	,220*	,019