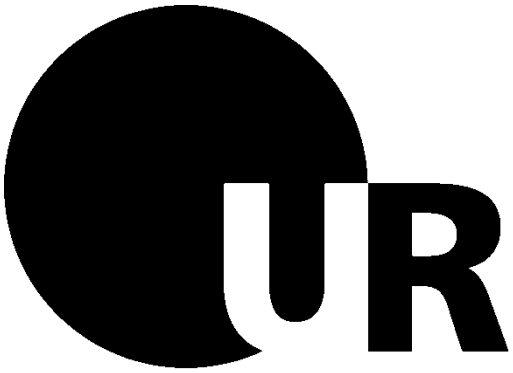
Immunomodulation by NOD2/CARD15 and Vitamin D_3



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To my parents

Aos meus pais

Table of Contents

LIST OF FIGURES ..... **IV**
LIST OF TABLES ..... **VII**
LIST OF ABBREVIATIONS ..... **VIII**
1. INTRODUCTION **1**
1.1. Hematopoietic stem cell transplantation **1**
1.2. Graft-versus-host disease (GvHD) **1**
1.2.1. Acute GvHD (aGvHD) 1
1.2.2. Chronic GvHD (cGvHD) 2
1.3. Role of innate immune cell responses in GvHD **3**
1.3.1. Importance of antigen-presenting cells (APCs) in GvHD 3
1.3.1.1. Origin of dendritic cells (DCs) 4
1.3.1.2. Tolerogenic dendritic cells 5
1.3.2. The role of pattern-recognition receptors in GvHD 6
1.3.3. NOD2 status and GvHD 7
1.3.4. Gut microbiome and GvHD 8
1.4. Role of the adaptive immune system in GvHD **10**
1.4.1. B cells in GvHD 10
1.4.2. Regulatory T cells (Tregs) in GvHD 11
1.5. Vitamin D **14**
1.5.1. Vitamin D metabolism 14
1.5.2. Vitamin D and immune function 16
1.5.3. Vitamin D status and GvHD 18
2. RESEARCH GOALS AND OBJECTIVES **20**
3. MATERIAL **21**
3.1. Equipment **21**
3.2. Consumables **22**
3.3. Media buffers and solutions **23**
3.4. Enzymes, kits and reagents for molecular biology **24**
3.5. Chemicals for cell culture **25**
3.6. Antibiotics **25**
3.7. Oligonucleotides for qRT-PCR **25**
3.8. Antibodies **26**
3.8.1. Western blot antibodies 26

3.8.2. Flow cytometry antibodies 26
3.8.2.1. Anti-mouse antibodies 26
3.8.3. Anti-human antibodies 27
3.9. Mice 28
3.10. Databases and software 29
4. METHODS 30
4.1. Cell culture 30
4.1.1. Isolation and culture of human monocytes 30
4.1.2. Macrophage culture 31
4.1.3. Dendritic cell culture 31
4.1.4. Mixed lymphocyte reaction 31
4.1.5. Cytokine analysis from culture supernatant 32
4.1.6. Enzymatic determination of lactate 32
4.1.7. Analysis of the oxygen content in cultures 32
4.1.8. Sorting of $CD4^{+}25^{-}$ T cells 33
4.1.9. Freezing and thawing of cells 33
4.1.10. Cell counting 33
4.2. Flow cytometry 33
4.2.1. Compensation 34
4.2.2. Surface staining 34
4.2.3. Intracellular staining 34
4.2.4. Blood sample preparation for flow cytometry 35
4.3. Molecular biological methods 35
4.3.1. RNA isolation 35
4.3.2. Polymerase chain reaction 35
4.3.3. DNA isolation 37
4.4. Biochemical methods 38
4.4.1. Protein isolation 38
4.4.2. SDS polyacrylamide gel electrophoresis 40
4.4.3. Western blot analysis 41
4.5. Mouse experiments 43
4.6. Microbiota analysis 44
4.7. Statistical analysis 44
5. RESULTS 45
5.1. Impact of NOD2 polymorphisms on the immune cell composition in peripheral blood of healthy donors 45

5.1.1. Impact of NOD2$^{-/-}$ on immune cell composition in mice 48
5.2. Impact of blood sampling and cell isolation method on the cell phenotype ... 49
5.2.1. CD16 downregulation: Importance of the recognized epitope 51
5.2.2. Effect of platelets and TGF-β on human monocytes 52
5.3. Impact of NOD2 on monocyte-derived macrophages 59
5.4. Impact of NOD2 on dendritic cell differentiation cultures 61
5.4.1. Dendritic cell cultures of donors with NOD2 WT SNP8 and SNP12 and 13 61
5.4.2. Effect of the NOD2-ligand MDP on monocyte-derived dendritic cells 64
5.4.2.2. Cytokine profile of Mo-derived DC *versus* MNC-derived DC 66
5.4.2.3. Oxygen consumption and lactate production of Mo-derived DC cultures 67
5.5. Impact of vitamin D_3 on immune cells 70
5.5.1. Effect of vitamin D_3 on human monocytes 70
5.5.2. Effect of Vitamin D_3 on human T cells 71
5.6. In vivo effects of vitamin D_3 75
5.6.1. Vitamin D_3 sufficient *versus* deficient mouse model 75
5.6.1.1. Impact of the serum 25(OH)D_3 level on immune cell composition 77
6. DISCUSSION & PERSPECTIVES 83
6.1. Impact of the NOD2 status on immune cell composition in human blood 83
6.1.1. Role of NOD2 in myeloid cell differentiation 84
6.1.1.1. NOD2 status and dendritic cell differentiation/maturation 84
6.1.1.2. NOD2 status and macrophage differentiation 85
6.1.2. Regulation of CD16 86
6.1.3. Role of NOD2 for monocyte activation 86
6.1.3.1. Impact of NOD2 on NF-κB signalling 87
6.1.4. Role of NOD2 ligands for cell activation and differentiation 88
6.2. Role of vitamin D_3 on immune regulation 90
6.2.1. Effect of vitamin D_3 on human T cells 91
6.2.2. *In vivo* effects of vitamin D_3 on mice 93
6.3. Impact of vitamin D_3 on microbiota 95
6.4. Perspectives 96
7. SUMMARY 99
8. ZUSAMMENFASSUNG 101
9. REFERENCES 104
PUBLICATIONS 118
ACKNOWLEDGMENT 119

List of Figures

Figure 1.1. The three phases of GvHD……..…….....2

Figure 1.2. Structure of NOD2/CARD15……...…..…7

Figure 1.3. Development of nTregs and iTregs……..……..11

Figure 1.4. Vitamin D metabolism…….…….....15

Figure 1.5. Effects of 1,25$(OH)_2D_3$ in various immune cells…….……..17

Figure 5.1. Impact of NOD2 SNP mutations on the blood cell composition of healthy donors……..……..…..…....46

Figure 5.2. Amount of $CD4^+$, and $CD4^+CD25^+$ and CD8 + T cells among living CD3+ cells in the blood of WT and donors with NOD2 variants…….…46

Figure 5.3. Amount of myeloid and plasmacytoid dendritic cells among living lineage negative HLA-DR^+ cells in the blood of WT, SNP8, 12 and 13 healthy donors……47

Figure 5.4. Amount of CD33+ cells among living leukocytes in the blood of WT, SNP8, 12 and 13 healthy donors …….....48

Figure 5.5. Impact of NOD2 expression on myeloid blood cells of WT and NOD2-/- mice.....49

Figure 5.6. Median fluorescence intensity (MFI) of CD14+16+ monocytes among living CD33+ cells in the blood of WT, SNP8, 12 and 13 healthy donors…….……50

Figure 5.7. Impact of the blood collection method on CD16 expression ……..……..51

Figure 5.8. Relative CD1 expression on monocyte using different antibody clones……52

Figure 5.9. Percentage of $CD14^+$ $CD16^+$ WT monocytes co-cultured with platelets or TGF-β……..…...53

Figure 5.10. Percentage of $CD14^+CD16^+$monocytes stimulated with or without MDP, TGF-β or LPS……..…….54

Figure 5.11. Percentage of $CD14^+$ monocytes stimulated with or without MDP, TGF-β or LPS…….…….55

Figure 5.12. Percentage of HLA-DR^+ monocytes stimulated with or without MDP, TGF-β or LPS …….…….....55

Figure 5.13. Cytokine secretion in monocytes stimulated with or without MDP, TGF-β and LPS or LPS+MDP……..…….…57

Figure 5.14. Effect of NOD2 mutations on iκBβ and phospho p38 expression…….....…….58

Figure 5.15. Expression of maturation markers in monocyte-derived macrophages from donors with NOD2 WT and NOD2 variants. …….....60

Figure 5.16. SNP13 donors displayed an impaired DC maturation…….…...…62

Figure 5.17. IL-10 production is similar in cultures from WT and NOD2 SNP donors…….....62

Figure 5.18. SNP12 and 13 donors showed enhanced activation in classical DCs.....63

Figure 5.19. IL-10 and IL-12 production is impaired in cultures from NOD2 SNP donors…….....64

Figure 5.20. MDP is incapable to induce the differentiation of human monocyte-derived dendritic cells (Mo-DCs).…….....65

Figure 5.21. Expression of tolerogenic DC markers after culture with LPS, MDP and 1,25$(OH)_2D_3$…….....66

Figure 5.22. IL10 and IL-12 secretion dendritic cells generated under different protocols. ……..…….....67

Figure 5.23. Oxygen consumption and lactate production of the Mo-derived DCs ……..68

Figure 5.24. Functional analysis of the generated DCs …….....69

Figure 5.25. Amount of FOXP3+ CD4+ cells after co-culturing with DCs matured either with MDP or LPS ……70

Figure 5.26. NOD2 mRNA expression in human monocytes…….....…….....71

Figure 5.27. Amount of FoxP3+ cells and cell proliferation in CD4+ T cell cultures. ……..…….....72

Figure 5.28. . FOXP3 induction by 1,25(OH)2D3 and 25(OH)D3 in comparison to TGF-β ……..…….....73

Figure 5.29. FOXP3 mRNA expression is increased upon TGF-β + 1,25 $(OH)_2D_3$ treatment ……..…….....73

Figure 5.30. Amount of 1,25$(OH)_2D_3$ in the culture supernatant of human CD4+ T cells. ……..…….....74

Figure 5.31. Cytokine profile of $CD4^{+}25^{-}$ T cells stimulated with TGF-β, $25(OH)D_3$ or $1,25(OH)_2D_3$.
…….....75

Figure 5.32. Serum vitamin D3 levels of BALB/c mice and CD1 mice at the age of 10 weeks…….…….……..….....76

Figure 5.33. Immune cell composition of BALB/c and CD-1 mice ……..…….…....77

Figure 5.34. Analysis of immune cell subpopulations in the blood, spleen and bone marrow of BALB/c and CD-1 mice .....……79

Figure 5.35. Analysis of immune cell subpopulations in the blood, spleen and bone marrow of BALB/c and CD-1 mice……..……80

Figure 5.36. Mice fed a vitamin D_3 deficient diet have higher microbiota diversity……..…...…81

Figure 5.37. The ten most abundant bacterial genera in mice stool samples.....82

List of Tables

Table 1.1. Properties of both natural and induced regulatory T cells……..….12

Table 4.1. Elutriation parameters and cell types……29

Table 4.2. Criteria for primer design…….……35

Table 4.3. Reaction mix used in qRT-PCR…….……36

Table 4.4. Program used for qRT-PCR…….……..……..……36

Table 4.5. Buffer A composition……37

Table 4.6. Buffer B composition……...……..38

Table 4.7. Buffer C composition……..…….....38

Table 4.8. SDS sample buffer composition…….……38

Table 4.9. SDS-PAGE stock solutions……..……..39

Table 4.10. SDS-PAGE gel mix solutions…….……..39

Table 4.11. Required buffers and solutions for SDS gel preparation…….…....39

Table 4.12. Western blot buffers…….....……41

Table 4.13. Composition of ECL solutions…….……..41

List of Abbreviations

aGvHD	acute Graft versus Host Disease
ANOVA	Analysis of variance
APCs	Antigen presenting cells
APS	Ammonium persulfate
BM	Bone Marrow
CARD	Caspase Recruitment Domain
cDNA	complementary DNA
cGvHD	chronic Graft- versus -Host Disease
CTLA4	Cytotoxic T-lymphocyte-associated Protein 4
DAPI	4',6-diamidino-2-phenylindole
DAMPs	Danger associated molecular patterns
Def.	Deficient
DNA	Deoxyribonucleic acid
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
dNTPs	2'-deoxyribonucleosid-5'-triphosphate
dsDNA	Double stranded DNA
ECL	Enhanced chemiluminescence
ECP	Extracorporeal Photophoresis
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
Foxp3	Forkhead Box Protein 3
GI	Gastro intestinal
GM-CSF	Granulocyte -monocyte colony-stimulating factor
GvHD	Graft versus Host Disease
GvL	Graft versus Leukemia
Gy	Gray
HANK´s BSS	Hank´s buffered saline solution
HLA	Human Leukocyte Antigen
HRP	Horse radish peroxidase
HSCs	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplantation
IDO	Indoleamine 2,3-dioxygenase

IFNγ	interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iTregs	induced Tregs
IκB	Inhibitor of kappa B
KDa	Kilodalton
LCs	Langerhans cells
LPS	Lipopolysaccharide
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight
MDP	Muramyl Dipeptide
MDSC	Myeloid-derived suppressor cells
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
NF-κB	Nuclear factor κB
NK	Natural Killer
NKT	Natural Killer T cell
NOD2	Nucleotide oligomerization domain 2
nTregs	natural Tregs
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBSC	Peripheral Blood Stem Cells
PCR	Polymerase chain reaction
PRR	Pathogen recognition receptor
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RT	Room temperature
rRNA	ribosomal RNA
s.e.m	standard error of mean
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
Suf.	Sufficient
TBI	Total Body Irradiation
TBS	Tris buffer saline
TBST	TBS + Tween 20
TCR	T Cell Receptor
TGF	Transformation Growth Factor
TLR	Toll Like Receptor

TNF	Tumor Necrosis Factor
Tregs	Regulatory T cells
UV	Ultraviolet

1. Introduction

1.1. Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation was originally conceived more than 60 years ago as a therapeutic option for irradiation injury. Later, this procedure was adopted as a method for treating several disorders, namely cancers and autoimmune diseases [1].
There are different types of hematopoietic stem cell transplantation (HSCT): Autologous, where the patient´s own stem cells are transplanted; allogeneic, using stem cells from a donor and syngeneic, where cells from an identical twin are used [2].
One of the most common complications after allogeneic HSCT (aHSCT) is the development of Graft-versus-host disease (GvHD).

1.2. Graft-versus-host disease (GvHD)

GvHD is still the most common life-threatening complication associated with HSCT[3]. It was first observed in 1956 in a murine model, where Barnes et al. demonstrated that irradiated mice which were infused with allogeneic bone marrow and spleen cells, recovered from irradiation injury and marrow aplasia but additionally developed several complications like diarrhea, weight loss and skin changes, due to a “secondary disease”. This condition was later recognized as Graft-versus-host disease [4]. GvHD can be considered both acute and chronic, depending on the timing of its occurrence.

1.2.1. Acute GvHD (aGvHD)

Acute GvHD generally occurs before day 100 after HSCT, but may also occur later. It is a reaction of donor immune cells against host tissues and affects the skin, liver, and gastrointestinal tract [5].
The pathophysiology of aGvHD is often described as a three-step process where the innate and adaptive immune system interact (Figure 1.1). In the first step, tissue damage occurs as a result of conditioning, releasing danger signals which induce pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β). This in turn leads to the activation of host antigen presenting cells (APCs). The activated APCs then stimulate donor T cells in the second step, also known as afferent phase. These T cell responses depend on the disparity between donor and recipient e.g. regarding human leucocyte antigen (HLA) or minor antigens.
In the last phase, or efferent phase, both innate and adaptive immune cells work synergistically to exacerbate T cell induced inflammation. Microbial products like lipopolysaccharide (LPS) leak through the damaged epithelium and continue to stimulate

mononuclear cells (monocytes, macrophages) leading to the release of inflammatory cytokines. As a result, further destruction of epithelial cells occurs especially in the gastrointestinal (GI) tract [4, 6].

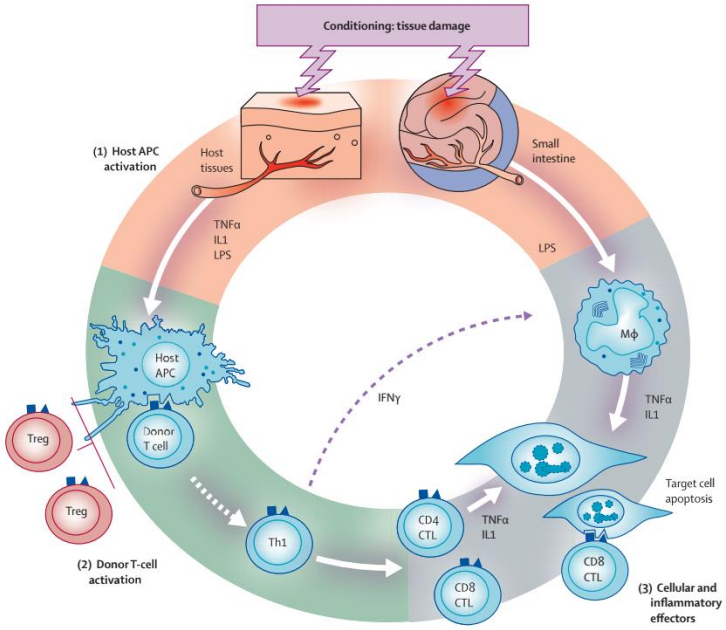

Figure 1.1. The three phases of aGvHD, according to Ferrara et al [6]. In phase I, chemotherapy or radiotherapy as part of transplant conditioning causes host tissue damage and release of inflammatory cytokines such as TNF, IL-1β, and IL-6, with resulting priming of host antigen-presenting cells (APCs). In phase II, host APCs activate mature donor cells, which then proliferate and differentiate. Release of additional effector molecules, such as TNF and IL-1β, mediates additional tissue damage. Lipopolysaccharide (LPS) that has leaked through damaged intestinal mucosa triggers further TNF production. The TNF can damage the tissue directly by inducing necrosis and apoptosis in the skin and gastrointestinal tract through either TNF receptors or the Fas pathway. TNF plays a direct role in intestinal GVHD damage, which further amplifies the damage in the skin, liver, and lung in a "cytokine storm." The process culminates in death of host cells through $CD8^+$ cytotoxic T cell-mediated apoptosis.

1.2.2. Chronic GvHD (cGvHD)

Chronic GVHD was initially defined as "late" GvHD syndrome that appears more than 100 days post-transplant, either as an extension of aGvHD (progressive onset GVHD), after a disease-free interval (quiescent cGvHD), or without preceding aGvHD (de novo cGvHD) [7].

The pathophysiology of cGvHD is poorly understood; therefore it remains one of the most significant complications of long-term survivors after aHSCT [8].

The absence of reliable animal models hampers the experimental study of this condition, although in the last decades some theories attempt to explain the pathophysiology of cGVHD: (1) thymic damage and the defective negative selection of T cells, (2) regulatory T cell deficiencies, (3) auto-antibody production by aberrant B cells, and (4) the formation of profibrotic lesions [9].

1.3. Role of innate immune cell responses in GvHD

1.3.1. Importance of antigen-presenting cells (APCs) in GvHD

Many cell types, including both hematopoietic and non-hematopoietic cells (e.g. endothelial cells), participate in the process of antigen presentation with distinct levels of efficiency. Hematopoietic APCs include B cells, monocytes, macrophages, as well as myeloid and plasmacytoid dendritic cells (pDCs). Besides antigen-presentation, APCs also are crucially involved in the production of (inflammatory) cytokines which regulate GvHD development [7].

Naïve B cells are absent from the skin and most mucosal sites, but instead they recirculate between blood and the secondary lymphoid organs. Here, they pick up antigens through specific B cell receptors for presentation on MHC class II and become competent APCs on interaction with $CD40L^+$ $CD4^+$ T cells [8].

Monocytes and macrophages are important players in inflammation. Their role includes phagocytosis, elimination of bacteria and restoration of tissue homeostasis [9, 10]. To fulfill their tasks, they can adopt different functional phenotypes dependent on the respective microenvironment [10]. Monocytes infiltrate into tissues where the polarizing stimuli to which they respond, result in macrophage or DC differentiation. Distinct macrophage and DC subsets have also been described *in vitro* [10-13]. Classical or M1-activated macrophages are anti-microbial, anti-tumorigenic and pro-inflammatory. This M1 phenotype plays a central role in chronic inflammation and autoimmunity. In contrast, alternative or M2 activation by IL-4 and/or IL-13 leads to differentiation of anti-inflammatory macrophages [10]. It is believed however, that these M1 and M2 subtypes represent extremes of a continuum of activation states found *in vivo* [7]. Monocytes and macrophages can also act as APCs and stimulate a secondary T cell response, but initiate a primary immune response in a weaker degree, which is the main function of the specialized APCs known as DCs. Importantly, DCs possess the capacity to pick up antigens in peripheral tissues and traffic to secondary lymphoid organs [14]. Therefore, DCs are largely effective at priming naïve T cells, although the extent to which they do so is greatly dependent on their origin and activation history [15].

Mouse studies have demonstrated that $CD4^+$ T cell-dependent (MHC-mismatched) aGvHD can be induced by either host or donor APCs [16-18]. Little is known about the role of DCs in cGvHD because of the lack of relevant mouse models. Both host and donor APCs have been implicated; skin cGvHD can be induced by either donor or host APCs, whereas donor APCs are dominant in intestinal cGvHD [19].

Generally, T cells have been the primary focus in the development of GvHD, but given the important role of DCs in its pathogenesis, APCs also represent an important target.

DCs could be manipulated using multiple approaches *in vivo* or *in vitro*, and the generation of tolerogenic DCs (tDCs) could be an interesting approach to regulate immunity and suppress GvHD [20].

1.3.1.1. Origin of dendritic cells (DCs)

DCs were first described in the mid-1970s by Ralph Steinman, who observed in the spleen a subpopulation of cells with a remarkable dendritic shape. It was soon appreciated that these cells existed in all lymphoid and most non-lymphoid tissues [21]. They are the most potent APC of the immune system and they are termed professional APC as a result of their exclusive ability to capture and to present antigens to naïve T cells.

They are part of the myeloid lineage of hematopoietic cells and arise from a precursor that can also differentiate into monocytes [22].

Although they share many common features, DCs are a heterogeneous cell population. Various cytokines and transcription factors are known to be responsible for the development of DC subpopulations. Depending on the subpopulation and the maturation state of these cells, they are able to induce either a broad cytotoxic immune response or induce immune tolerance which is of great importance within the context of autoimmunity [15].

Epithelial DCs, named Langerhans cells (LCs) are present exclusively in epithelial tissues (epidermis, mucosa) and they express receptors for antigen uptake, e.g. Fcγ and Fcε, as well as CD1a, a molecule involved in the presentation of glycolipids to T cells.

Interstitial DCs, identified in the interstitium of most tissues, are characterized by the expression of CD68 and factor XIIIa, but lack LC markers. These cells display similarities with monocyte-derived DCs.

Monocyte-derived DCs can be generated *in vitro* in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Allegedly, in the course of inflammation *in vivo*, this type of differentiation occurs physiologically. Blood monocytes can also differentiate into DCs during transendothelial migration [23].

In the human blood, different DC subsets have been identified, namely myeloid dendritic cells (mDCs), characterized by the expression of HLA-DR and CD11c, and plasmacytoid dendritic cells (pDCs) which have a plasma cell-like morphology and lack CD11c expression [23].

Under normal (healthy) conditions, DCs exist in an immature or steady state and their main function is to maintain immune tolerance by keeping adaptive immune cells from attacking host cells that possess “self” antigens. However, if DCs encounter “non-self” entities in the periphery, they opsonize them, process their antigens for cross-

presentation, migrate to the lymph nodes, and prime naïve T cells for the respective antigen. iDCs (immature DCs) exhibit continuous endocytic activity and therefore continuously present “self” antigens to T cells. Nevertheless, T cells are not polarized toward an effector state but are rather polarized to facilitate tolerance or immunosuppression. Such immunotolerance is accomplished due to the complete lack of stimulatory signals provided by the iDC [24]. On the other hand, when DCs encounter pathogens, they switch to a mature state exhibiting strong phenotypic and functional stimulation. Mature DCs are characterized by the high expression of costimulatory molecules, namely CD80, CD83 and CD86. The simultaneous presence of phenotypic maturation ligands and the appropriate cytokine profile of the DC (e.g. high IL-12 secretion) helps to activate effector functions in interacting T cells, thus polarizing them for antigen-specific elimination of the “non-self” entity [24].

1.3.1.2. Tolerogenic dendritic cells

Tolerogenic DCs are characterized by the downregulation of different costimulatory molecules such as CD80 and CD86 as well as the upregulation of inhibitory receptors like PDL-1, PDL-2, ILT3, ILT4 and TIM3. It is believed that ILT3 and ILT4 directly inhibit proliferation of T cells and drive $FOXP3^+$ Treg expansion [25].

Tolerogenic DCs also exhibit a special cytokine profile. This type of DC produces higher amounts of IL-10 and reduced amounts of IL-12. As IL-12 is crucial for the induction of a Th1 response, tolerogenic DCs are more likely to induce the generation of Th2 cells or Tregs [26].

Indoleamine 2, 3-dioxygenase (IDO), an immune checkpoint enzyme is also expressed by tolerogenic DCs. IDO metabolizes tryptophan to kynurenine and its expression by DCs has been implicated in the inhibition of T cell proliferation and survival. Furthermore, it has been reported that IDO expression by DCs promotes the development of Tregs [27].

Tolerogenic DCs have the capability to induce long-lasting tolerance to transplanted allogeneic grafts and suppress the development of autoimmune diseases [28]. Hence, tolerogenic DCs represent a subtype of polarized DCs which inhibit immune activation.

Diverse factors have been reported to support the generation of tolerogenic DCs with low antigen-presenting capability. These factors belong to different categories, such as cytokines (e.g. IL-10 and TGF-β), vitamins (e.g. vitamin D) and drugs (e.g. dexamethasone or rapamycin) [29].

With their regulatory capacities tolerogenic DC seem to be a promising tool to mitigate GvHD

In fact, the suppression of DC function is under investigation in the prevention and treatment of human GvHD while ensuring the conservation of graft-versus-leukemia effects [19].

1.3.2. The role of pattern-recognition receptors in GvHD

Specific receptors on innate immune cells, called pattern recognition receptors (PRRs), sense pathogen-associated molecular patterns (PAMPs), such as LPS and damage-associated molecular patterns (DAMPs), like high mobility group box 1 (HMGB-1).

Danger signalling is transmitted through PRRs when they bind PAMPs and DAMPs. Several signalling molecules, such as toll-like receptor (TLR), Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), and retinoic acid-inducible gene 1 (RIG-1) signalling are recognized.

TLRs are a family of PRRs and are expressed on a variety of cells derived from both hematopoietic and non-hematopoietic lineages [30]. There are several TLRs identified (TLR1-13) and they interact with different ligands. For example, LPS binds to TLR4 whereas flagellin binds to TLR5.

Another known PRR subtype refers to NLRs. These receptors are commonly expressed by immune cells and some non-immune cells. NOD1 and NOD2 are the most extensively investigated NLRs in GvHD. NOD1 and NOD2 recognize different kinds of peptidoglycan (PGN) fragments from the bacterial cell wall. NOD1 binds to diaminopimelate-containing N-acetyl glucosamine-N-acetylmuramic acid (GluNAc-MurNac) tripeptides from gram-negative bacterial PGN, whereas NOD2 binds to muramyl dipeptide (MDP) that is produced by all bacteria [30].

Some studies have shown that TLR4 mutations lead to the reduction of GvHD risk in mice and humans [12, 13]. In a study using TLR4-knockout mice ($TLR4^{-/-}$) as bone marrow and splenocyte cell graft donors or recipients, the occurrence of GvHD symptoms and mortality were delayed compared to wild-type ($TLR4^{+/+}$) mice [12]. It has also been reported that TLR4 expression is upregulated in patients with cutaneous cGvHD [14]. Nevertheless, there are also both mice and human studies showing no significant impact of TLR4 mutations in the outcome of HSCT [31].

In the clinical setting, single nucleotide polymorphisms (SNPs) of the gene encoding NOD2 are associated with a higher GvHD incidence [32-34]. SNPs are defined as mutations with an allele frequency of at least 1% in a given population and can have modest biological effects [22]. NOD2 has been shown to have an inhibitory effect on GvHD by suppressing the function of APCs and favouring the generation of regulatory T cells (Tregs) [35].

1.3.3. NOD2 status and GvHD

The NOD2/CARD15 gene encodes the NOD2 protein that functions as a sensor for MDP. It takes part in the regulation of inflammatory responses through intracellular pathways involving nuclear factor κB (NF-κB) and IκB kinase [36].

The NOD2 protein (Figure 2) contains a series of carboxy-terminal leucine-rich repeats (LRR), as well as a central nucleotide-binding domain (NBD; also known as NOD domain). In addition, the amino-terminal domain contains tandem CARDs, which interact with receptor interacting protein 2 (RIP2) upon NOD2 stimulation [37]. Activation of RIP2 leads to subsequent steps that ultimately result in activation attended by nuclear translocation of NF-κB and transcription of its target genes [38].

The ligand sensing occurs at the LRR domain of NOD and the recognition of MDP is believed to trigger a conformational change in the protein, promoting a NBD-mediated oligomerization of NOD2 [39].

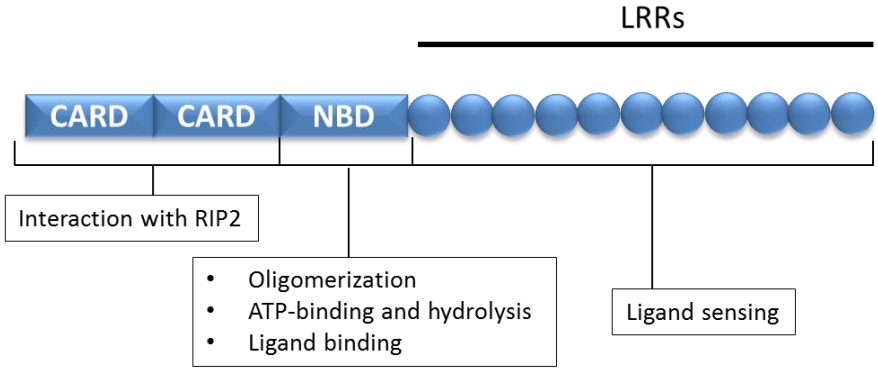

Figure 1.2. Structure of NOD2/CARD15 gene. NOD2 contains tandem N-terminal CARDs that interact with the CARD of receptor-interacting protein 2 (RIP2). NOD2 contains a central nucleotide-binding domain (NBD), which binds ATP and mediates NOD oligomerization and carboxy-terminal leucine-rich repeats (LRRs which are important for ligand sensing. Adapted from [39]**.**

NOD2 was identified several years ago and is one of the well-studied members of the NLR family. Initially, NOD2 expression was reported to be relatively tissue specific and restricted to APCs such as monocytes, macrophages, DCs, and certain epithelial cells [40]. More recently, it has been reported that basal levels of NOD2 expression are usually quite low in other cell types (myeloblastic cells, dental pulp) but can be induced by a variety of inflammatory signals such as LPS), TNF-α, interferon (IFN)-γ and 1,25-dihydroxylvitamin D3 ($1,25(OH)_2D_3$) [40].

The association of SNPs of the NOD2 gene with a number of inflammatory pathologies, including Crohn´s disease (CD), and Blau syndrome, highlights its pivotal role in host-pathogen interactions and the inflammatory response [41]. More than 10 years ago, SNPs in the NOD2 gene locus have been associated with GvHD [33, 34]. Holler *et al*. found an association between SNPs (alleles 8, 12, and 13) of the NOD2 gene in the

donor or host and a higher incidence of GvHD, as well as an increased transplant-related mortality. These results were partially confirmed by Elmaagacli et al [32] who found a relation between NOD2 status and outcome after allogeneic stem cell transplantation only if both recipient and donor carried NOD2 SNPs. Although there is still no consensus regarding the role of the NOD2 status with regard to the outcome of HSCT [42, 43], MDP sensing by hematopoietic cells seems to be of crucial importance for the intestinal immune homeostasis [44] and it appears that NOD2 SNPs of patients receiving aHSCT influence post-transplant immunity, even though the underlying mechanism is still not clear [45].

1.3.4. Gut microbiome and GvHD

It is estimated that about one thousand microbial species live in symbiosis with the human gut [46]. These bacterial communities, collectively called microbiome, play an important role in the regulation of metabolic processes and also appear to be critical for the optimal function of the immune system [46]. Several studies have already demonstrated that particular species of microbiota can contribute to the induction of different immune cell types such as $Foxp3^+$ regulatory T cells, suggesting a close relationship between the microbiome and the immune system [47].

The gut microbiome is regulated by different mechanisms: On the one hand, the gastrointestinal epithelial barrier, in addition to its absorptive role, is involved in the regulation of microbial colonization and mucosal immune responses. On the other hand, specialized intestinal epithelial cells (IECs), such as the secretory goblet cells and Paneth cells, regulate bacterial species composition through the production of a mucous layer and the secretion of antimicrobial proteins, such as defensins. These antimicrobial peptides are produced by cells in the course of innate defence and serve as signals which initiate, mobilise, and amplify adaptive immune defences by the host. Defensins can be classified in two sub-families. Based on their tertiary structure, there are α- and β-defensins. While human α-defensins are mostly expressed by neutrophils, β-defensins are secreted by epithelial cells of the skin and mucosae. Besides their anti-microbial activity, accumulating data arose in the past decade indicating that defensins have extended functions in human patho(physio)logy. Indeed, defensins appear as modulators of the adaptive immune system and angiogenesis, key mediators of wound healing and determinant players in male fertility [48].

In early studies, mice transplanted in germ-free conditions or treated with antibiotics developed significantly less GvHD, demonstrating a negative role for bacteria and the microbiome in the GvHD-related mortality [49, 50]. Also, human studies using

prophylactic gut decontamination demonstrated prevention in the development of aGvHD [51].

More recent studies indicate that the gut microbiome is altered after HSCT and the composition of the microbiome is important for transplantation outcome. Patients who develop GvHD have an altered microbiome, with loss of dominance of Clostridiales species replaced by a higher number of Lactobacillales or Enterobacteriales [47]. Results from a retrospective study showed that *Clostridium difficile* infection (CDI) is strongly associated with aGVHD and increased nonrelapse mortality in aHSCT patients [69]. Whether dysbiosis is just an epiphenomenon due to the use of antibiotics is still not clear but the loss of commensal bacteria correlates with GvHD severity and pathogenesis in human patients [52]. Furthermore, the importance of the microbiome in the setting of GvHD was demonstrated in animal studies [47], showing that the balance between specific gut bacteria plays a pivotal role in transplantation outcome. Namely, the expansion of Lactobacillales and loss of Clostridiales was found to be present in a GvHD mouse model [52].

The protective effect of commensal bacteria can be explained by the secretion of certain bacterial metabolites. In a study conducted by Reddy et al., the authors demonstrated that the bacterial metabolite butyrate is consistently decreased in the intestinal tissue after allogeneic stem cell transplantation. Butyrate is mainly produced by Clostridium, Eubacterium, and Butyrivibrio bacteria genera and it has been shown that butyrate improves intestinal epithelial barrier function, thereby mitigating GvHD [53]. Another possible mechanism connecting dysbiosis and GvHD is the ability of certain bacteria to promote T cell differentiation. For instances, Clostridiales bacteria have been reported to be upregulators of colonic regulatory T cells (Tregs) [54]. This may suggest that GvHD could deplete anti-inflammatory cell populations by reducing the abundance of Clostridiales.

In line with the hypothesis that the intestinal microbial balance is of the outmost importance for the transplantation outcome, fecal microbiota transplantation (FMT) could be an option for transplantation patients. FMT refers to infusion of a fecal suspension from a healthy individual into the GI tract of a patient to restore “healthy” intestinal microbiota and cure disease [55]. In recent years, FMT has emerged as an effective method for the treatment of CDI. However, there is risk that fecal suspension could contain harmful bacteria, viruses, and parasites. An alternative solution to this problem is to screen the beneficial bacteria from stool and infuse these bacteria as a cocktail into the gastrointestinal tract [56].

Although the importance of the gut microbiome in the regulation of the (intestinal) immune system is indisputable, further studies are needed to clarify the relation between

gut microbiome and complications after transplantation. These could pave the way for new therapeutic options for the prevention of GvHD.

1.4. Role of the adaptive immune system in GvHD

In GvHD pathophysiology, the interplay between innate and adaptive immune system is of utmost importance.

After the initial response of the innate immune system to microbial products and molecules released by cellular damage, the adaptive immune response is initiated.

Both donor $CD4^+$ and $CD8^+$ T cells have crucial roles in the pathophysiology of GvHD. Therefore, the most effective approach for GvHD prevention still is the depletion or suppression of donor T cells [57].

However, recent animal studies suggest that B cells might also play an important role in the biology of GvHD [58]. Further evidence for the involvement of B cells in GvHD pathogenesis comes from reports of successful treatment of GvHD with B cell depletion therapies [59].

1.4.1. B cells in GvHD

Little is known about the role of B cells in the pathogenesis of aGvHD. In mice, B cell depletion results in a decreased incidence of aGvHD [58]. Further support for a role of B cells in aGvHD comes from several recent clinical observations: Encouraged by reports of the effectiveness of B cell depletion in the treatment of cGvHD, Kamble et al treated patients with aGvHD, who were refractory to multiple immunosuppressive drugs, with rituximab, a chimeric monoclonal antibody directed against the B cell-specific CD20 antigen. They reported that three patients who received rituximab showed a complete remission of aGvHD. Hence, B cell depletion might also be effective for the prevention of aGvHD. Rituximab administered as part of a myeloablative or nonmyeloablative conditioning regimen or given shortly before or after transplantation results in lower-than-expected rates of GvHD [60].

Even though these results indicate that B cells are involved in the development of GvHD, most likely due to their role as APCs, other reports show that host B cells can also have protective effects as B cell-deficient mice experienced more severe aGvHD than wild-type mice. In this experimental model IL-10 production by recipient B cells is responsible for the suppression of aGvHD [61].

The involvement of B cells in cGvHD seems to be more straightforward since both preclinical and clinical data suggest an important role of B cell dysregulation in cGvHD pathogenesis and treatment [35]. Post-transplant administration of rituximab appears to reduce the rate of cGVHD in preliminary studies [62].

1.4.2. Regulatory T cells (Tregs) in GvHD

Tregs are a subset of $CD4^+$ T cells with immunosuppressive activity. They are characterized by the expression of the IL-2R α-chain (CD25) and the transcription factor FOXP3 [63]. However, identification of human Tregs is problematic since these and other markers (CD25, CTLA-4, CD127, FOXP3) seem to represent general T cell activation markers [74].

Tregs are essential for the maintenance of self-tolerance, regulation of the peripheral T cell pool, and contribute to tolerance in solid organ transplantation and protection from GvHD lethality in bone marrow transplantation (BMT) models [64].

It is believed that there are two different subsets of $CD4^+$ Treg cells. The natural occurring Tregs (nTregs) develop in the thymus. During negative selection, nTreg cells upregulate FOXP3 during self-antigen recognition [65]. nTreg cells are responsible for maintaining immune homeostasis and tolerance to self-antigen by inhibiting self-reactive T cells in the periphery. The other subset of $CD4^+$ Tregs has been named "induced" Tregs (iTregs). These cells are generated in the periphery when conventional T cells are activated in the presence of TGF-β and IL-2 [66] (Figure 1.3).

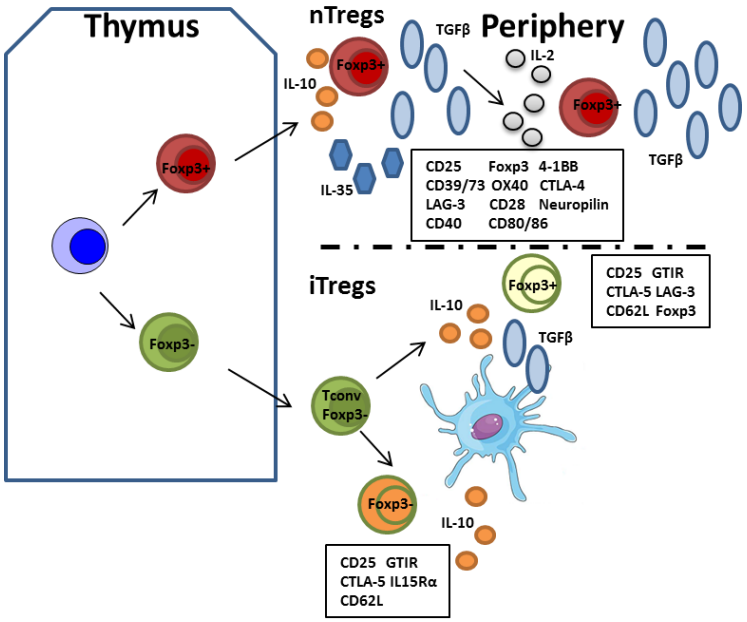

Figure 1.3. Development of nTregs and iTregs. Adapted from [66]. nTregs (top) differentiate from naïve conventional T cells to Foxp3+ Tregs in the thymus. In the periphery, natural Tregs express a number of cell surface markers, indicated in the box below the depiction of the natural Treg. However, none of these cell surface markers are unique to Tregs as they are also found on activated conventional T cells. Natural Tregs utilize the cytokines IL-10, IL-35 and TGFβ to exert their suppressive effects upon conventional T cells. TGFβ and IL-2 have also been shown to be important to the maintenance and fidelity of the Treg signature. iTregs (bottom) can be generated from conventional T cell precursors. Once in the periphery, naïve conventional T cells can be induced to become Foxp3− Tr1 cells or Foxp3+ Th3 cells via IL-10 and/or TGFβ secreted by APCs such as dendritic cells and macrophages. These induced Tregs share similar cell surface markers as natural Tregs. Foxp3+ induced Tregs can accumulate in the gut through upregulation of CCR9 and α4β7 via TGFβ and retinoic acid produced by CD103+ dendritic cells.

Induced Tregs can be also generated *in vitro* by activating naïve T cells with the proper culture conditions. T cells activated with either antigen or anti-CD3/28 beads in the presence of TGF-β and IL-2 are capable to up-regulate Foxp3, and also display other features used to characterize regulatory T cells, such as a suppressive function.

The *in vitro*-generated iTregs are attractive in the clinical setting [67], since they can be generated in large numbers, facilitating the adoptive transfer of these cells into the recipients.

The two Treg subsets differ in their main antigen specificities, in the T-cell receptor signal strength and also in the co-stimulatory requirements needed for their generation (Table 1). Nonetheless, it is still not clear whether iTregs exist *in vivo***.**

Table 1.2. Properties of both natural regulatory T cells (nTregs) and induced regulatory T cells (iTregs). Adapted from [68].

	nTregs	**iTregs (TGF-β+ IL-2)**
Principal antigen specificities	Self	Foreign
Generation		
IL-2 and TGFβ required	No	Yes
CD28-dependent	Yes	No
CTLA-4-dependent	No	Yes
Maintenance		
IL-2 and TGFβ required	Yes	Yes
Stability		
IL-6 can convert to Th17 cells	Yes	No
Can block other cells from producing IL-17	No	Yes
Suppression		
IL-6 can block suppressive activity	Yes	No

There is evidence that IL-6 is capable of converting nTregs to Th17 cells, while iTregs induced by IL-2 and TGF-β are resistant to this cytokine and retain their suppressive function at inflammatory sites. Thus, nTregs and iTregs may have different roles in the adaptive immune response [68].

Since GvHD is characterized by the loss of tolerance and the occurrence of autoimmune manifestations, it seems reasonable to assume that Tregs may have a critical role in the pathophysiology of GvHD [67]. In fact, animal studies have already demonstrated that a deficiency in Tregs contribute to both aGvHD and cGvHD [69, 70].

Given the critical role of Tregs in maintaining tolerance, several groups postulated that the adoptive transfer of Tregs should ameliorate GvHD [70, 71]. The first study was performed in mice by Taylor et al. [70]. In this study, the authors depleted both $CD4^+$ $CD25^+$ T cells from the donor and recipient before transplantation and observed that the depletion of this cell population was associated with worsening of GvHD. In contrast, the adoptive transfer of $CD4^+$ $CD25^+$ nTreg cells along with the marrow graft resulted in the amelioration of disease. Hoffmann and co-workers [72] demonstrated that freshly isolated $CD4^+$ $CD25^+$ T cells from unprimed animals can rescue recipients from lethal GvHD induced by $CD4^+$ $CD25^-$ T cells after allogeneic transplantation. That protection from lethality is partially dependent on IL-10 produced by transplanted $CD4^+$ $CD25^+$ T cells.

The role of Tregs in GvHD mitigation has also been demonstrated in humans: Rezvani and colleagues demonstrated that a high number of $CD4^+$ $CD25^+$ $FOXP3^+$ T cells in the donor graft were associated with a reduced risk of GvHD [73]. Also, they found that low nTreg counts early after aHSCT were associated with an increased risk of GvHD.

Since one of the challenges in nTreg therapy is the reduced frequency of these cells in peripheral blood, there have been several attempts in order to expand this population. It is possible to expand nTreg cells through *in vivo*-stimulation of nTreg proliferation or through *ex vivo*-expansion [74]

Based on the fact that nTregs express high levels of IL-2Rα, Kennedy-Nasser et al [75] treated 16 pediatric patients with ultralow doses of IL-2 three times per week, starting from day 7 or 30 after transplantation for 12 weeks, and they observed an increase in $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs from baseline. These cells were able to suppress alloreactive responses *in vitro*, indicating functionality.

The first clinical trial using Tregs to suppress acute/chronic GvHD in patients was reported eight years ago. This “first-in-man-study” described the adoptive transfer of *ex vivo*-expanded $CD4^+$ $CD25^+$ $CD127^-$ Tregs in one patient with cGvHD and another with aGvHD after HSCT with an HLA-identical sibling donor [76] . The therapy resulted in significant alleviation of the symptoms and reduction of pharmacologic immunosuppression in the case of cGvHD, while in the case of grade IV aGvHD it only transiently improved the condition. Since then, several other studies have been conducted [77] including the use of umbilical cord blood as a source of Tregs for *in vitro*-expansion [78].

1.5. Vitamin D

The classical, hormonal actions of vitamin D are related with calcium and bone homeostasis. Vitamin D enhances absorption of calcium in the small intestine and stimulates osteoclast differentiation and calcium reabsorption from bone. Furthermore, Vitamin D promotes mineralization of the collagen matrix [79].

Over the last decade it has become evident that the role of Vitamin D in human health is not limited to bone homeostasis. Several studies have already suggested that Vitamin D deficiency is associated with the development of cardiovascular diseases, various types of cancer and autoimmune disorders, such as type 1 diabetes mellitus (T1D), multiple sclerosis (MS) and inflammatory bowel disease (IBD) [80].

With the finding that the Vitamin D receptor (VDR) and enzymes important in Vitamin D metabolism such as 1-α-hydroxylase (CYP27B1) are expressed in different cell types like epithelial cells, and cells of the immune system, the interest of studying the impact of this hormone raised, particularly in the field of human immunology.

1.5.1. Vitamin D metabolism

There are three possible sources of vitamin D: nutritional, UVB-dependent endogenous production and supplements.

In humans, the main source is through UVB exposure. Only a small part is derived from the diet. There are very few natural products that can be used as vitamin D source. These include fatty fish such as salmon, sardines and cod liver oil [79].

Due to its dependence on UVB exposure, vitamin D levels can fluctuate depending on the season of the year, latitude, skin pigmentation and lifestyle [79].

In the human skin, cholecalciferol is synthesized from 7-dihydrocholesterol by ultraviolet irradiation (Figure 1.4).

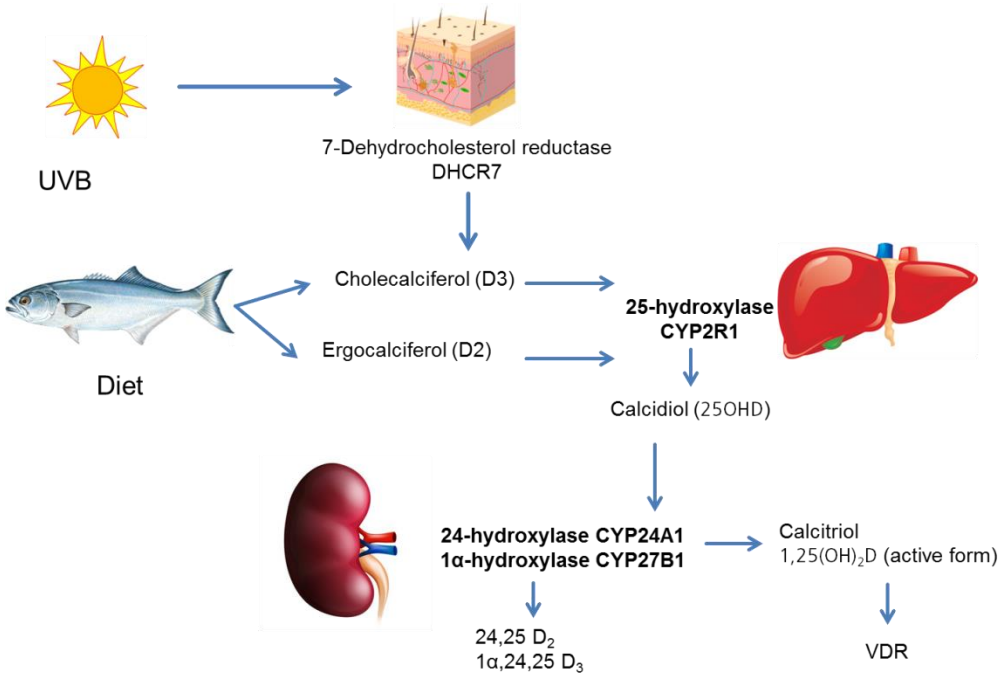

Figure 1.4. Vitamin D metabolism. Cholecalciferol is synthesized from 7-dihydrocholesterol by UV irradiation, or can be obtained in small amounts from the diet. In the liver, cholecalciferol is hydroxylated to calcidiol 25(OH)D_3 through the action of CYP2R1. Once in the kidney, 25(OH)D_3 is further converted into its active form, 1,25(OH)$_2D_3$.

Cholecalciferol is biologically inactive. It binds to transport proteins such as vitamin D binding proteins or albumin and enters the circulation. In the liver cholecalciferol is hydroxylated, a process catalysed by the enzymes CYPR1 and CYP27A1, resulting in the production of the inactive form 25-hydroxyvitamin D_3 (25(OH)D_3), which represents the main circulating form of vitamin D. This form is also the most stable and is used as a parameter to define one person's vitamin D status [79, 81].

In the kidney, 25(OH)D_3 is further converted to the biologically active form of vitamin D, calcitriol (1,25(OH)$_2D_3$) by the action of the enzyme 1-α-hydroxylase (CYP27B1).

Calcitriol levels are regulated in a renal negative feedback loop, including the inhibition of CYP27B1 by high calcitriol levels and stimulation of CYP24A1 (24-hydroxylase), an enzyme that metabolizes calcitriol to calcitroic acid, the inactive, water soluble form, which is then excreted in the bile. Although the circulation levels of 1,25(OH)$_2D_3$ levels are mainly determined by renal CYP27B1, other cell types such as immune cells which express this enzyme are also capable of converting 25(OH)D_3 into its biologically active form. The production of 1,25(OH)$_2D_3$ by macrophages and dendritic cells may lead to high local concentrations of 1,25(OH)$_2D_3$ which are assumed to be important for immunomodulation [81].

1.5.2. Vitamin D and immune function

Early evidence for the importance of vitamin D for the immune system dates from the 19th century. Cod liver oil was used as a treatment for infections such as consumption, a condition these days known as tuberculosis. Tuberculosis patients were also sent to sanatoriums, where therapy included sunlight exposure. It was believed that sun exposure directly kills *Mycobacterium tuberculosis* bacteria [79]. In the meantime we know that the effect of both, sunlight and cod liver oil, is due to vitamin D.

M. tuberculosis is a strong inducer of CYP27B1 and VDR in monocytes and macrophages after pathogen recognition by toll-like receptors. $1,25(OH)_2D3$ in turn can induce antimicrobial peptides such as cathelicidin and β-defensins [92] [79].

Human cathelicidin (LL37) is produced as a precursor protein called hCAP18, which upon cleavage releases LL37. Further processing can occur in the skin which results in the release of shorter peptides. hCAP18 is widely expressed in the immune system and also in a variety of epithelial cells. Beside tuberculosis, low $25(OH)D_3$ serum levels have been associated with various infectious diseases, such as influenza, chronic obstructive pulmonary disease and allergic asthma and were found to be significantly lower in critically ill septic patients. It has also been shown that low $25(OH)D_3$ serum levels correlates with decreased concentrations of antimicrobial peptides such as cathelicidin [82].

Taken together, these findings support the idea that the vitamin D status regulates antimicrobial protein levels that are critical for the control of infection.

Besides its effect on antimicrobial protein regulation, vitamin D also has other effects on the activation of immune cells [83]. In the hematopoietic system, VDR is expressed in various hematopoietic precursors as well as in monocytes, thymocytes and activated B and T cells [80]. Some studies with VDR-knock-out (VDR-KO) mice show that these animals produce normal number and proportion of blood cells, demonstrating that vitamin D signalling is not necessary for the differentiation of the hematopoietic cell repertoire [84]. Although the number of cells is not changed, it has been shown that there are changes in the cytokine profile in VDR-KO mice that may affect T-helper (Th) immune responses indicating that $1,25(OH)_2D3$ may regulate immune cell function [84]. Nevertheless there are studies stating that VDR also seems to be critical for the development of invariant natural killer T (iNKT) cells, a subset of T cells with a regulatory role in autoimmunity and infection [85]. Cantorna and co-workers have demonstrated that $CD8^+$ T cells and iNKT cells are targets for vitamin D [85]. VDR-KO mice have significant less iNKT cells, and the few iNKT cells present in the periphery are functionally defective. Like iNKT cells, there are also less CD8αα/TCRαβ precursors in the thymus of VDR-KO animals. CD8αα T cells are mainly present in the gut, where they have a role in

preserving tolerance and suppress inflammation [85]. Taken together, these results show that VDR expression is compulsory for the development of both iNKT and CD8αα/TCRαβ cells.

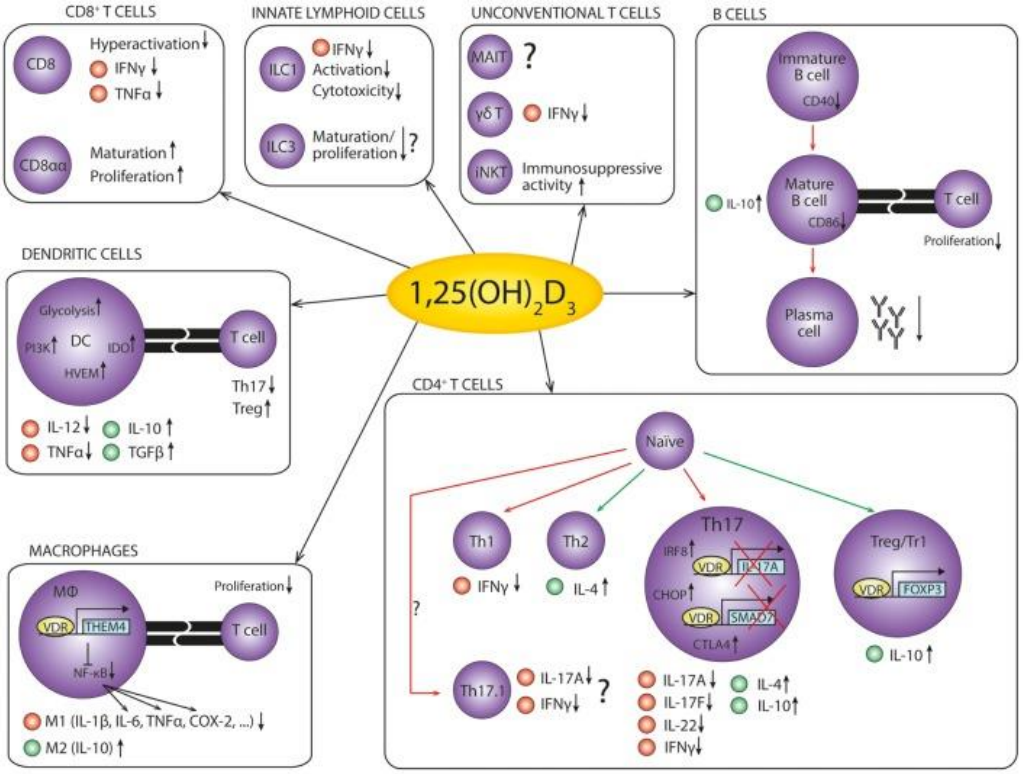

Figure 1.5. Effects of $1,25(OH)_2D_3$ on various immune cells. Adapted from [80].

Dendritic cells differentiated in the presence of vitamin D remain in an immature-like tolerogenic state. This can be accessed by the cytokine profile of these cells. Pro-inflammatory cytokines like IL-12 and TNF are reduced and anti-inflammatory cytokines like IL-10 are increased. Due to their maturation status, these tolerogenic DCs (tDCs) are less capable of stimulating T cell proliferation and cytokine production of pro-inflammatory T cells, whereas they induce the differentiation of regulatory T cells.

It was demonstrated that $1,25(OH)_2D3$ promotes the anti-inflammatory M2 phenotype of macrophages and is capable to induce the differentiation of monocytes into macrophages *in vitro* [86].

Although the effect of $1,25(OH)_2D3$ on B cells is still not completely understood, several reports suggest that vitamin D reduces the proliferation of B cells, induces their apoptosis and inhibits immunoglobulin class switching [87]. There is also evidence that vitamin D decreases antibody production [83].

For a long time it was believed that the effect of vitamin D on T cells was indirect and mediated exclusively by antigen-presenting cells. However, it has become clear now that upon activation, T cells upregulate the VDR, making this cell type another important target for vitamin D.

Vitamin D treatment has been shown to have an impact on different T cell subtypes., namely by inducing regulatory T cells [88]. When $1,25(OH)_2D_3$ is added to T cells, it inhibits the differentiation of pro-inflammatory Th1 cells by suppressing IFN-γ and favouring Th2 response. In VDR KO, the Th1 response is also impaired.

1.5.3. Vitamin D status and GvHD

In an optimal stem cell transplantation scenario, the donor immune system would destroy the malignant cells without harming the healthy recipient tissues. In order to prevent or treat GvHD, immune suppressive medication is required, which is crucial to (re)balance the immune response in patients.

Since vitamin D has already been evidenced as an important immunomodulatory hormone, this suggests a possible significance in the outcome of HSCT [77].

In fact, the relationship between vitamin D serum levels and GvHD outcome has already been investigated by a number of groups [89-92].

Kreutz *et al*. reported an analysis of $25(OH)D_3$ and $1,25(OH)_2D_3$ serum levels in 48 patients undergoing allogeneic bone marrow transplantation (BMT) and found a decline in both metabolite levels in the course of transplantation [89].

In a different study, Glotzbecker and colleagues demonstrated that vitamin D deficiency before HSCT is associated with an increased risk of cGVHD [90].

VDR genes are polymorphic in the human population and their variants have also been a subject of investigation in patients undergoing HSCT [93-95].

Cho and co-workers hypothesized that polymorphisms of the VDR gene could affect clinical outcomes of allogeneic HSCT [96]. Three VDR gene polymorphisms (***Bsm***I G>A, ***Apa***I G>T, and ***Taq***I T>C) were genotyped in 147 patients who underwent HLA-matched sibling aHSCT. Frequencies of infection, GvHD, overall survival (OS), and disease-free survival (DFS) were compared according to genotypes and haplotypes. In this study they found a correlation between polymorphisms at the *Taq*I cleavage site, where heterozygous patients had better DFS and OS than TT homozygote patients.

Also, they found that recipients having two copies of the “A” allele for the *Apa*I polymorphism had lower rates of aGvHD and infection. Homozygosity of the “A” allele is associated with higher VDR activity and in a study performed by Middleton *et al.* [95] a similar effect was reported: a solid trend toward reduced aGvHD in recipients with the AA genotype.

Bogunia-Kubik and co-workers also published a study regarding VDR polymorphisms and HSCT, but included an analysis of patients receiving cells from unrelated donors. Here, they found an association between the FF genotype (associated with higher VDR activity) and the outcome after HSCT. If both donor and recipient had the FF genotype,

the recipient experienced higher risk of GvHD development. Interestingly, GvHD risk based on VDR polymorphisms depends on whether they are present in the donor or recipient.

So far, data suggest that both VDR and vitamin D may play a role in immune reconstitution and immune surveillance in transplant patients. Prospective studies are needed to investigate the effects of vitamin D supplementation, e.g. with $1,25(OH)_2D3$ analogues, on HSCT outcome [97]. In HSCT recipients, supplementation could promote better immune function in the reconstituted immune system and help to prevent the deleterious effects of GvHD.

2. Research Goals and Objectives

GvHD is still the most common major life-threatening complication associated with allogeneic hematopoietic stem cell transplantation (aHSCT). Previous data demonstrate that single nucleotide polymorphisms of the NOD2/CARD15 receptor are associated with increased risk of GvHD. Furthermore, vitamin D has emerged as a central player in the immune system, with its deficiency being implicated in the pathogenesis of several diseases, including the development of GvHD.

The aims of this dissertation were to clarify the role of NOD2 status for the regulation of the immune response, namely by studying the immune cell composition in peripheral blood of healthy donors differing in their NOD2 status. Also, the effects of muramyl dipeptide (MDP), the natural ligand of NOD2, were studied in monocyte/MNC-derived dendritic cell cultures.

Moreover, the effect of vitamin D on human T cells with particular interest in the generation of inducible regulatory T cells was investigated *in vitro*. In parallel, the *in vivo*-effects of vitamin D levels were also studied, using an animal model of vitamin D sufficiency *versus* deficiency to examine the impact of vitamin D in the immune cell composition of blood, spleen and bone marrow of the mice. Additionally, the impact of vitamin D levels on murine gut microbiota was analysed.

3. Material

3.1. Equipment

Autoclave	Technomara, Fernwald, Germany
Balance LP1200S	Sartorius, Göttingen, Germany
Bioanalyzer 2100	Agilent Technologies, Böblingen, Germany
Biofuge fresco	Heraeus, Osterode, Germany
CASY Cell Counter	Innovatis/Roche, Basel, Switzerland
Centrifuge Megafuge 3.0R	Heraeus, Osterode, Germany
Centrifuge Sigma 2	Sartorius, Göttingen, Germany
Electrophoresis equipment	Biometra, Göttingen, Germany
Electrophoresis equipment (PFGE)	Biostep, Jahnsdorf, Germany
ELISA plate reader	MWG Biotech, Ebersberg, Germany
EVOS Cell Imaging System	Life Technologies, Carlsbad, CA, USA
FACS Calibur flow cytometer	BD Biosciences, Franklin Lakes, NJ, USA
Forceps	Aesculap, Tuttlingen, Germany
Heat sealer	Eppendorf, Hamburg, Germany
Hemocytometer	Marienfeld, Lauda-Königshofen, Germany
Incubators	Heraeus, Hanau, Germany
Laminar Flow Cabinet Clean Air	Telstar, Woerden, The Netherlands
LSR II flow cytometer	Becton Dickinson, Heidelberg, Germany
LSR Fortessa	Becton Dickinson, Heidelberg, Germany
Microscopes	Zeiss, Jena, Germany
Multifuge 3S-R	Heraeus, Osterode, Germany
Multipipettor Multipette plus	Eppendorf, Hamburg, Germany
NanoDrop 1000	Thermo Fisher Scientific, Schwerte, Germany
PCR Thermocycler PTC-200	MJ-Research/Biometra, Oldendorf, Germany
pH Meter	Knick, Berlin, Germany
pH Meter, portable	Hanna Instruments, Kehl am Rhein, Germany
Picofuge	Heraeus, Osterode, Germany

Pipetboy	Integra Biosciences, Fernwald, Germany
Pipettes	Gilson, Milddleton, WI, USA
Pulsed field gel electrophoresis	GE Healthcare, Chalfont St Giles, UK
Realplex Mastercycler epGradient S	Eppendorf, Hamburg, Germany
Rocking plattform HS250	IKA Labortechnik, Staufen, Germany
Rotilabo® mini centrifuge	Carl Roth, Karlsruhe, Germany
SenSorDish- Reader	PreSens, Regensburg
Sonifier 250	Branson, Danbury, USA
Sonorex Ultrasonic Bath	Branson, Danbury, CT, USA
Speed Vac	Thermo Fisher Scientific, Hudson, NH, USA
Thermomixer	Eppendorf, Hamburg, Germany
Vortexer	Scientific Industries Ink., Bohemia, NY, USA
Water purification system	Millipore, Eschborn, Germany
Waterbath	Julabo, Seelstadt, Germany

3.2. Consumables

Cell culture flasks	Costar, Cambridge, MA, USA
Cell culture plates	Eppendorf, Hamburg, Germany
Cell culture plates, cell repellent	Greiner Bio-One, Kremsmünster, Austria
Cell scrapers	Sarstedt, Nümbrecht, Germany
Cell strainer (70 µm, 100 µm)	Falcon, Heidelberg, Germany
Combitips for Eppendorf multipette	Eppendorf, Hamburg, Germany
Cryo tubes	Corning, Corning, NY, USA
ep dualfilter T.I.P.S®	Eppendorf, Hamburg, Germany
Heat sealing film	Eppendorf, Hamburg, Germany
Hyperfilm™ ECL	GE Healthcare, Chalfont St Giles, UK
Micro test tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Micropore filters	Sartorius, Göttingen, Germany
Microtiter plates (6, 12, 24, 96 wells)	Costar, Cambridge, MA, USA
Microtiter plates for ELISA	Costar, Cambridge, MA, USA

OxoDishes (24 well)	PreSens, Regensburg, Germany
PCR plate Twin.tec 96 well	Eppendorf, Hamburg, Germany
Petri dish	Falcon, Heidelberg, Germany
Pipette tips	Eppendorf, Hamburg, Germany
Plastic pipettes	Costar, Cambridge, MA, USA
Polystyrene test tubes	Falcon, Heidelberg, Germany
Polystyrene test tubes with cell strainer cap	Corning, Corning, NY, USA
Scalpels (No. 10), disposable	Feather, Osaka, Japan
Syringe Filters, sterile	Sartorius, Göttingen, Germany
Syringes and needles	Becton Dickinson, Heidelberg, Germany
Teflon foils	Heraeus, Hanau, Germany
Tissue culture dish	Falcon, Heidelberg, Germany
Tubes (5 ml, 15 ml, 50 ml, 225 ml)	Falcon, Heidelberg, Germany
Whatman® Chromatography Paper	Sigma-Aldrich, St. Louis, MO, USA

3.3. Media buffers and solutions

2-Mercaptoethanol	Gibco/Life Technologies, Carlsbad, CA, USA
Acrylamide	Carl Roth, Karlsruhe, Germany
APS	Merck Millipore, Billerica, MA, USA
Aqua	Braun, Melsungen, Germany
Bovine serum albumine	Sigma-Aldrich, St. Louis, MO, USA
CasyTON	Roche, Basel, Switzerland
DMSO	Sigma-Aldrich, St. Louis, MO, USA
FACS clean	BD Biosciences, Franklin Lakes, NJ, USA
FACS flow	BD Biosciences, Franklin Lakes, NJ, USA
FACS rinse	BD Biosciences, Franklin Lakes, NJ, USA
Fetal calf serum	Gibco/Life Technologies, Carlsbad, CA, USA
Hydrogen peroxide	Merck, Darmstadt, Germany
Hydrochloride acid	Carl Roth, Karlsruhe, Germany
Isopropanol	Braun, Melsungen, Germany aun

L-Alanyl-L-Glutamine	Merck Millipore, Billerica, MA, USA
Methanol	Thermo Fisher Scientific, Waltham, MA, USA
Nuclease-free water	Gibco/Life Technologies, Carlsbad, CA, USA
PBS	Sigma-Aldrich, St. Louis, MO, USA
RPMI 1640	Gibco/Life Technologies, Carlsbad, CA, USA
TEMED	Sigma-Aldrich, St. Louis, MO, USA
Tween 20	Sigma-Aldrich, St. Louis, MO, USA

3.4. Enzymes, kits and reagents for molecular biology

Agilent RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, CA, USA
BD™ Comp Beads	BD Biosciences, Franklin Lakes, NJ, USA
Bio-Rad DC protein assay	Bio-Rad, Munich, Germany
CD4 Microbeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Collagenase IV	Worthington, Lakewood, NJ, USA
DAPI	Sigma-Aldrich, St. Louis, MO, USA
Dynabeads Human T-Activator CD3/CD28	ThermoFisher, Massachusetts, USA
DNeasy Blood & Tissue Kit	Qiagen, Hilden, Germany
dNTPs	Roche diagnostics, Mannheim, Germany
DuoSet ELISA Kits	R&D Systems, Minneapolis, MN, USA
FcR-Blocking reagent mouse	Miltenyi Biotec, Bergisch Gladbach, Germany
FoxP3 transciption factor staining buffer set	eBioscience, San Diego, CA, USA
GM-CSF	Berlex, Seattle, USA
Human interkeukin 2 (IL-2)	Promokine, Heidelberg, Germany
Human interkeukin 4 (IL-4)	Promokine, Heidelberg, Germany
M-MLV Reverse Transcriptase	Promega, Madison, WI, USA
Proteinase K	Roche, Mannheim, Germany
QuantiFast SYBR® green PCR Kit	Qiagen, Hilden, Germany

Random Decamers	Ambion/Life Technologies, Carlsbad, CA,USA
RNAlater™	Qiagen, Hilden, Germany
RNase-free DNase Set	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
SDS	Sigma-Aldrich, St. Louis, MO, USA

3.5. Chemicals for cell culture

Lipopolysaccharide (LPS)	Enzo Life Sciences, Farmingdale, NY, USA
Muramyl dipeptide (MDP)	InVivogen, Toulouse, France
25-hydroxyvitamin D_3 ($25(OH)D_3$)	Sigma-Aldrich, St. Louis, MO, USA
1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$)	Biozol, Eching, Germany

3.6. Antibiotics

Penicillin and streptomycin for primary cell culture were purchased from Gibco Life Technologies, Carlsbad, California, USA.

3.7. Oligonucleotides for qRT-PCR

Primers were designed using UCSC genome browser and PerlPrimer software. Unmodified, HPLC-purified oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Gene	**Primer sequence (sense and antisense)**
FoxP3 (mouse)	5´CCCATCCCCAGGAGTCTTGC 3´ 5´ACCATGACTAGGGGCACTGTA 3´
18S (mouse)	5'-ACCGATTGGATGGTTTAGTGAG-3' 5'-CCTACGGAAACCTTGTTACGAC-3'

3.8. Antibodies

3.8.1. Western blot antibodies

Antibodies	Species	Dilution	Molecular weight	Manufacturer
iKB	Rabbit	1:1000	45 kDa	Santa Cruz Biotechnology,
Phospho-p38	Rabbit	1:1000	43 kDa	Dallas, TX, USA
β-Actin	Rabbit	1:2000	42 kDa	Sigma-Aldrich, St. Louis, MO, USA
Rabbit-HRP	Goat		1:2500	Dako, Glostrup, Denmark

3.8.2. Flow cytometry antibodies

3.8.2.1. Anti-mouse antibodies

Specificity	Isotype	Conjugation	Clone	Manufacturer
Biotin	Mouse IgG1	PerCP	Bio3-18E7	Miltenyi Biotec
CD4	Rat IgG2a κ	FITC	RM4-5	eBioscience
CD8	Rat IgG2b κ	APC-Cy7	53-6.7	Biolegend
CD11b	Rat IgG2b κ	Pe-Cy7	M1/70	BD
CD19	Rat IgG2a κ	PE	6 D5	Biolegend
CD25	Rat IgG1 λ	Pe-Cy7	PC 61.5	eBioscience
FoxP3	Rat IgG2a κ	FITC	FJK-16s	eBioscience
Gr1	Rat IgG2b κ	APC	RB6-8C5	BD
Helios	Hamster IgG	PE	22F6	Biolegend
H2kb	Mouse IgGa κ	Biotin	AF6-88.5	Biolegend
NK1.1	Mouse IgGa κ	BV421	PK136	Biolegend
TCRβ	Hamster IgG	APC-Cy7	H57-597	Biolegend

3.8.3. Anti-human antibodies

Specificity	Isotype	Conjugation	Clone	Manufacturer
CD1a	Mouse IgG1,ƙ	PE	SFCI19Thy1A8	Beckman Coulter
CD3	Mouse IgG1,ƙ	FITC	SK7	BD
CD4	Mouse IgG1,ƙ	PE	RPA-T4	BD
CD8	Mouse IgG1,ƙ	APC-H7	SK1	BD
CD11c	Mouse IgG1,ƙ	PerCP	BU15	Abnova
CD14	Mouse IgG2a,ƙ	Horizon 450	M5E2	BD
CD14	Mouse IgG2b,ƙ	PE	MϕP9	BD
CD15	Mouse IgG1,ƙ	Pacific Blue	W6D3	Biolegend
CD16	Mouse IgG1,ƙ	APC	3G8	Biolegend
CD16	Mouse IgG1,ƙ	APC	B73.1	Biolegend
CD16	Mouse IgG1,ƙ	APC	VEP	Miltenyi Biotec
CD16	Mouse IgG1,ƙ	APC	REA423	Miltenyi Biotec
CD16b	Mouse IgG1,ƙ	APC	REA589	Miltenyi Biotec
CD19	Mouse IgG1,ƙ	FITC	HIB19	eBioscience
CD19	Mouse IgG1,ƙ	PerCP	SJ25C1	BD
CD21	Mouse IgG1,ƙ	APC	B-ly4	BD
CD25	Mouse IgG1,ƙ	Pe-Cy7	M-A251	BD
CD25	Mouse IgG1,ƙ	APC	2A3	BD
CD32	Mouse IgG1,ƙ	PE	FUN-2	Biolegend
CD33	Mouse IgG1,ƙ	PE	WM53	Biolegend
CD39	Mouse IgG2b,ƙ	PE	TU66	BD
CD45	Mouse IgG1,ƙ	PerCP	2D1	Biolegend
CD45RA	Mouse IgG2b,ƙ	Pe-Cy7	HI100	Biolegend
CD56	Mouse IgG2a,ƙ	FITC	MEM188	eBioscience
CD62P	Mouse IgG1,ƙ	PE	Ak4	Biolegend

CD73	Mouse IgG1,ƙ	FITC	AD2	BD
CD80	Mouse IgG1,ƙ	FITC	BB1	BD
CD83	Mouse IgG1,ƙ	FITC	HB15a	Beckman Coulter
CD85j (ILT2)	Mouse IgG2b,ƙ	Pe-Cy7	GI/75	Biolegend
CD85k (ILT3)	Mouse IgG1,ƙ	APC	ZM4.1	Biolegend
CD86	Mouse IgG1,ƙ	APC	2331(FUN-1)	BD
CD127	Mouse IgG1,ƙ	BV 510	HIL-7R-M21	BD
CD197 (CCR7)	Mouse IgG2a,ƙ	FITC	150503	BD
CD209 (DC-Sign)	Mouse IgG2b,ƙ	PE	120507	R&D
CD273(PD-L2)	Rat IgG2a, κ	APC	TY25	Biolegend
CD274(PD-L1)	Mouse IgG2b,ƙ	Pe-Cy7	29E.2A3	Biolegend
CD366(TIM3)	Mouse IgG1,ƙ	Pe-Cy7	F38-2E2	Biolegend
CX3R1	Rat IgG2b,ƙ	Pe-Cy7	2A9-1	Biolegend
HLA-DR	Mouse IgG2a,ƙ	APC-H7	G46-6	BD
IgD	Mouse IgG2a,ƙ	PE	IA6-2	BD
IgM	Mouse IgG1,ƙ	FITC	G-20-127	BD
Lin Cocktail	Mouse IgG1,ƙ	FITC	L27	BD

3.9. Mice

Strain	Provider
BALB/c	Charles River Laboratories
CD-1	Provided by the Central Animal Facility University Hospital Regensburg
C57BL/6	Provided by Dr. Petra Hoffmann, University Hospital Regensburg
NOD2$^{-/-}$	

3.10. Databases and software

The following databases and software tools were used to design experiments and process data.

CellQuest Pro	BD, Heidelberg, Germany
EndNote X7	Thomson Reuters, New York, NY, USA
ExonMine	http://www.imm.fm.ul.pt/exonmine/
FACSDiva	BD, Heidelberg, Germany
FlowJo version 9.5.3	FlowJo,LLC, Ashland, OR, USA
GraphPad Prism version 5.02	GraphPad Software, La Jolla, CA, USA
ImageLab version 4.0	Bio-Rad, Munich, Germany
Microsoft Excel 2003/2007/2010	Microsoft Deutschland GmbH
PerlPrimer version 1.1.14	http://perlprimer.sourceforge.net/
PubMed	http://www.ncbi.nlm.nih.gov/entrez
SenSorDish-Reader Software	PreSens, Regensburg, Germany
USCS Genome Browser	http://www.genome.ucsc.edu

4. Methods

4.1. Cell culture

4.1.1. Isolation and culture of human monocytes

4.1.1.1. Isolation of human monocytes

Peripheral blood mononuclear cells (PBMCs) were separated by leukapheresis of healthy volunteer donors, followed by density gradient centrifugation over Ficoll/Hypaque. Monocytes were then isolated from mononuclear cells (MNCs) by counterflow centrifugation elutriation. Elutriation was performed using a J6M-E centrifuge equipped with a JU 5.0 elutriation rotor and a 50 ml flow chamber (Beckman, Munich, Germany). After system sterilization with 6% H_2O_2 for 20 minutes, the system was washed with PBS. Following calibration at 2500 rpm and 4 °C with Hank's BSS, MNCs were loaded into the system at the flow rate determined after calibration.
Fractions were collected according to the right flow rate (see table 4.1). The last fraction collected represented the monocytes, the largest cells within the MNCs. The monocyte yield was donor dependent, normally ranging from 10 to 20%. The purity of the monocyte fraction was then determined by flow cytometry and varied from 85 to 95%.

Table 4.1. Elutriation parameters and cell types

Fraction	Volume (ml)	Flow rate (ml/min)	Main cells isolated
Ia	1000	52	Platelets
Ib	1000	57	
IIa	1000	64	
IIb	500	74	B and T lymphocytes, NK cells
IIc	400	82	
IId	400	92	
III	800	130	Monocytes

4.1.1.2. Culture and stimulation of human monocytes

For the TGF-β experiments, the isolated monocytes were plated ($2x10^6$ cells/ml), in 6-well plates with a cell-repellent surface (Greiner BioOne, Kremsmünster, Austria) in RPMI 1640 supplemented with 2% AB serum, 2 mmol/l L-Glutamin (Biochrom, Berlin), 50 U/ml Penicillin and 50 µg/ml Streptomycin (both Gibco, Darmstadt) .

Cells were stimulated overnight with TGF-β, LPS, MDP or combination of the substances.
For the platelet co-culture, monocytes ($2x10^6$ cells/ml) were co-incubated overnight with platelets ($20x10^6$) isolated from elutriation fraction Ia.

4.1.2. Macrophage culture

Macrophages were obtained by culturing monocytes at a density of $1x10^6$/ml in RPMI medium supplemented with 2% AB serum in teflon bags for 7 days. After this period, the cells were harvested by placing the teflon bag in the fridge for 30 minutes and after gently rubbing the bag. The cells were decanted to a falcon tube. Afterwards, the collected cells were analysed for cell viability and surface markers by means of flow cytometry.

4.1.3. Dendritic cell culture

For the "classical" protocol, immature monocyte-derived dendritic cells (iDCs) were generated by culturing $10x10^6$ elutriated monocytes in 15 ml ($0,67x10^6$ cells/ml) RPMI 1640 medium supplemented with 10% FCS, 2 mol/l L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin, 144 U/ml IL-4 and 280 U/ml GM-CSF. Cells were incubated for 5 days at 37°C with 5% CO_2 and 95% relative humidity. On day 5, iDCs were stimulated with 100 ng/ml LPS, 100 ng/ml MDP or a combination of both substances for 48 hours. On day 7, mature dendritic cells were harvested.
When the "fast" protocol was applied, iDCs were generated by culturing $10x10^6$ elutriated monocytes in 15 ml ($0,67x10^6$ cells/ml) RPMI 1640 medium supplemented with 10% FCS, 2 mol/l L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin 114 U/ml IL-4 and 225 U/ml GM-CSF. Alternatively, $40x10^6$ MNCs/15 ml ($2,8x10^6$/ml) were incubated for 24 hours at 37°C with 5% CO_2 and 95% relative humidity, and then stimulated for 48 hours with the same stimuli used in the "classical" protocol.
When generating MNC-DC in 96-well plates, the fast protocol was applied, culturing $1x10^6$ MNC/100 µl/well.

4.1.4. Mixed lymphocyte reaction

10,000 DCs were cocultured with 100,000 allogeneic T lymphocytes, isolated from elutriation´s IIb fraction, in RPMI containing 5% AB serum, L-glutamine (2 mmol/L), penicillin (50 U/mL), and streptomycin (50 mg/mL). Cells were cultured in round bottom 96-well plates, in a final volume of 200 µl (100 µl T lymphocytes + 100 µl DCs). On day 5, supernatants were harvested and frozen at -20°C until analysis. On day 7, cells were harvested, counted and analyzed by flow cytometry.

For the vitamin D_3 experiments, T cells (isolated from elutriation's IIb fraction either by magnetic bead sorting ($CD4^+$ cells) or FACS sorting ($CD4^+CD25^-$T cells) were cocultured with Dynabeads Human T-Activator CD3/CD28 (0,1 bead/cell or 1 bead/cell), in RPMI medium containing 2% AB serum, 100U/ml IL-2, L-glutamine (2 mmol/L), penicillin (50 U/mL), and streptomycin (50 mg/mL) for 3 or 4 days. Afterwards, cells were harvested, counted and analyzed by means of flow cytometry. Cells were further cultured and analyzed at the indicated periods of time.

4.1.5. Cytokine analysis from culture supernatant

After harvesting the cells, the supernatants were frozen and cytokines present in culture supernatants were measured by means of sandwich ELISA. The capture antibody is attached to a solid phase (microtiter plate). After blocking unspecific binding-sites, the sample is added and the antigen of interest will bind. Afterwards, the plate was washed and the detection antibody added, recognizing the antigen of interest. The next step involves the addition of an enzyme-linked secondary antibody that will bind to the detection antibody. Finally, the substrate is added, and is enzymatically converted to a detectable form. The fluorescence is measured using a microtiter plate reader and the concentration of the antigen is quantified using a standard curve.

4.1.6. Enzymatic determination of lactate

To determine lactate concentrations, supernatants were harvested and frozen at -20°C until analysis. For the measurement, an ADVIA 1650 analyser was used at the Department of Clinical Chemistry (University Hospital Regensburg, Germany).

4.1.7. Analysis of the oxygen content in cultures

Oxygen content of cell cultures was measured with using the SensorDish-Reader system (PreSens, Regensburg). This system allows a non-invasive measurement of O_2 content in the culture, using 24-well plates that contain a sensor spot at the bottom of each well. The sensor spot contains a luminescent dye that is excited by the SensorDish Reader placed below the multidish, and its luminescence lifetime is detected non-invasively through the transparent bottom. The luminescence of the dye depends on the oxygen partial pressure, as molecular oxygen quenches/deactivates the luminescent indicator. Measured values are afterwards converted by the software with the help of the stored calibration data.

For the analysis of the oxygen content of DC cultures (section 5.3.1.3), monocytes ($1x10^6$) or MNC ($2x10^6$) were seeded in 24-well OxoDishes and incubated at 37°C for 24

hours. Afterwards, cells were stimulated with LPS, MDP (100 ng/ml) or combination and placed onto the SensorDish Reader for 48 hours.

4.1.8. Sorting of $CD4^+25^-$ T cells

Peripheral blood mononuclear cells (PBMCs) were separated by leukapheresis of healthy volunteer donors, followed by density gradient centrifugation over Ficoll/Hypaque. Afterwards, $CD4^+$ cells were separated using $CD4^+$ T cell isolation kit from Miltenyi Biotec. For some experiments (section 5.5.1) the resulting population was further separated to obtain $CD4^+CD25^-$ T cells by FACS with a FACSAria sorter.

4.1.9. Freezing and thawing of cells

For freezing, *circa* 40 million cells were resuspended in 1 ml RPMI medium. Freezing medium (80% FCS and 20% DMSO) was added in a 1:1-ratio and slow-frozen in cryo-freezing vials for short term storage at -80°C in an isopropanol-filled container (Nalgene) for 24 hours. For long term storage, the samples were then transferred to liquid nitrogen (-196°C).
Frozen cells were thawed at room temperature and directly added to serum containing medium. After centrifugation, the cells were resuspended in fresh medium.

4.1.10. Cell counting

Cell number and viability were determined using the CASY system, an electric field multi-channel cell counting system. This system quantifies cells passing a measuring pore where cells are exposed to a low voltage electrical field. Based on the resistance signal generated, cells can be differentiated, since dying or dead cells have a lower resistance due to their higher membrane permeability, whereas living cells generate high resistance signals due to their intact membrane structure.

4.2. Flow cytometry

Flow cytometry is a technique that allows detection, counting and sorting of individual cells. Cell components are stained with fluorochrome-labeled antibodies and then excited by a laser to emit light at different wavelengths. This technique measures various characteristics of single particles flowing individually in a stream of fluid. The scattering of light in different angles can discriminate cells regarding differences in size (forward scatter, FSC) and granularity (side scatter, SSC), while fluorescence from the labeled antibodies allows the identification of various cell surface and cytoplasmic antigens. This approach makes flow cytometry a powerful tool for cell analysis in a short period of time, giving both qualitative and quantitative information about complex populations[98].

Different fluorochrome-conjugate antibodies against the antigens of interest were used in 6-colour measurements performed using a BD LSR II cytometer, equipped with a red, blue, violet and UV laser. The staining procedure was performed on ice to preserve cell viability.
Dead cells were excluded by 4',6-diamidino-2-phenylindole (DAPI), a DNA-binding dye that enters cells with damaged membranes.
Analysis of the generated flow cytometry data was performed with the FlowJo software.

4.2.1. Compensation

All fluorochromes possess their particular excitation and emission spectra. The excitation spectra refer to the range of light wavelength that adds energy to a fluorochrome, causing it to emit light in another wavelength, the emission spectrum. In a flow cytometer there are bandpass filters that select the right ranges of excitation and emission wavelengths. However, when more than one marker is expressed in a single cell, their emission spectra may overlap, giving false results. This is called spillover and occurs whenever the fluorescence emission of one fluorochrome is detected in a detector designed to measure signal from another fluorochrome. To avoid this, a process called compensation is required, allowing the correction of the detected spillover [99].
BD compbeads were used to determine compensation values, according to manufacturer's instructions. To determine cell autofluorescence, unstained cells were used.

4.2.2. Surface staining

For surface staining of antigens, 0,5-1x10^6 million cells were placed in flow cytometry tubes and centrifuged at 525 g at 4°C for 4 minutes with 1 ml flow cytometry buffer. Afterwards, the cells were incubated with the proper antibody amount for 30 minutes at 4°C in the dark. Next, cells were centrifuged twice with 1 ml of flow cytometry wash buffer and the pellet was resuspended in 300 µl flow cytometry buffer.
The cells were measured on the same day, after adding 10% of DAPI for discrimination between living and dead ones.

4.2.3. Intracellular staining

For the intracellular staining of antigens, cells had to be permeabilized, using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), following manufacturer's instructions. Briefly, 0.5 ml of FIX/Perm diluent (diluted 1:4) was added to the cells, vortexed and incubated for 45 minutes at 4°C. The cells were then centrifuged three times with Perm solution (diluted 1:10 with H_2O) and the pellet was incubated with the

proper antibody amount for 30 minutes at 4°C. After the incubation time, the cells were washed twice with 1 ml of Perm solution and one time with flow cytometry buffer. In the end, the pellet was resuspended in 300 µl of flow cytometry buffer and the data acquired at the flow cytometer.

4.2.4. Blood sample preparation for flow cytometry

The erythrocytes of 1 ml of blood collected from heparin, hirudin, EDTA and citrate tubes were lysed using 20 ml ACK lysis buffer (0.155 M NH_4Cl, 0.1 M $KHCO_3$ and 0.1 mM EDTA, diluted 1:6 with H_2O) for 5 minutes at room temperature. The reaction was stopped by adding 25 ml RPMI medium and cells were centrifuged. The procedure was repeated about 2 times, until the pellet was colourless. Cell pellets were resuspended in 1-50 ml medium (volume dependent on pellet size) and filtered via 100 µm cell strainers. Alternatively, PBMCs from the collected blood were separated by density gradient centrifugation over Ficoll/Hypaque.

4.3. Molecular biological methods

4.3.1. RNA isolation

For RNA isolation, cells were harvested, centrifuged at 350 g for 7 minutes, the dry pellet resuspended in RLT Buffer containing 1% 2-Mercaptoethanol and then sheared 10 times using a 1 ml syringe. Procedures were performed on ice and RNA lysates were frozen at -80°C for storage. The subsequent isolation of total cellular RNA was performed using the RNAeasy Mini Kit, according to manufacturer´s instructions (QUIAGEN). RNA concentration was determined using a NanoDrop 1000 spectrophotometer.

4.3.2. Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique used in molecular biology that aims the amplification of DNA fragments using a DNA template, a DNA polymerase and sequence-specific oligonucleotide primers which flank the DNA fragment of interest. Here, real-time quantitative PCR (RT-qPCR) was used in order to quantify the expression level of specific genes. Therefore, total cellular RNA had to be converted into copy DNA (cDNA) by reverse transcription.

4.3.2.1. Reverse transcription

In order to determine gene expression levels, total RNA was transcribed into cDNA using a reverse transcriptase from Moloney Murine Leukemia (M-MLV), random decamer primers and 2'-deoxyribonucleosid-5'-triphosphates (dNTPs). The reaction was performed in a PCR thermocycler by adjusting the volume of 1 µg of total RNA to 13 µl

nuclease-free ddH_20, mixing 1 µl random decamers (Promega) and 1 µl dNTPs (10 mM) on ice. Secondary structures of RNA were dissolved by 5 minutes incubation in the thermocycler at 65°C followed by immediate incubation on ice for 1 minute. After mixing with 4 µl M-MLV buffer (5x; Promega), samples were incubated at 42°C for 2 min. Reverse transcription started upon addition of 1 µl M-MLV (50 min, 42°C) and was stopped by heat inactivation of the enzyme (15 min, 70°C). cDNA samples were stored at -20°C.

4.3.2.2. Primer design

The specific primers used to detect the gene of interest by means of RT-qPCR were designed using PerlPrimer software and the USCS genome browser obeying the criteria presented in table 4.2.

Table 4.2. Criteria for primer design

GC content	40-60%
Melting temperature (T_m)	65-68°C
Primer lenght	20-28 bp
Amplicon size	70-150 bp
Position	On 2 adjacent exons

4.3.2.3. Real-time quantitative PCR

Real-time quantitative polymerase chain reaction (RT-qPCR) is a technique that allows gene expression quantification using a DNA-binding dye that intercalates double-stranded DNA causing more fluorescence when bound. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured at each cycle. The higher the initial number of DNA molecules in the sample, the faster the fluorescence will increase during the PCR cycles. This means that if a sample contains more targets, the fluorescence will be detected in earlier cycles. The cycle in which fluorescence can be detected is termed quantitation cycle (Cq) and is the basic result of RT-qPCR: lower Cq values mean higher initial copy numbers of the target. In the present work, the QuantiFast SYBR Green (manufacturer) was used. The reactions were prepared in 96-well PCR plates at a volume of 10 µl/well with a reaction mix illustrated in table 4.3. All samples were pipetted in triplicates and a positive control, used for the generation of a standard curve was pipetted in duplicates.

Table 4.3. Reaction mix used in RT-qPCR

QuantiFast SYBR Green-mix	5µl
cDNA (1:10 in H_2O_{USB})	1 µl
Sense primer	0.5 µl
Antisense primer	0.5 µl
H_2O_{USB}	3 µl

After plate preparation, the RT-qPCR was performed in a Realplex Mastercycler EpGradient S according to the program depicted in table 4.4.

Table 4.4. Program used for RT-qPCR

Process	Temperature	Duration	
Initial denaturation	95°C	5 min	
Denaturation	95°C	8 sec	45 cycles
Annealing/elongation	60°C	20 sec	
	95°C	15 sec	
Melting curve	65°C	15 sec	
	65-95°C	10 min	

Specific amplification of the expected PCR product was controlled by the pattern of the melting curve. By means of the slope of the standard curve and the y-axis intercept, the amount of cDNA was calculated by the Realplex software. The amount of messenger RNA (mRNA) which corresponds to the calculated amount of cDNA was finally normalized to that of *18S* rRNA.

4.3.3. DNA isolation

For the NOD2 SNP analysis, PBMCs were isolated and preserved in liquid nitrogen until analysis. Subsequently, genomic DNA was extracted using the DNeasy Blood & Tissue Kit from Quiagen, according to manufacturer´s instructions.

4.3.3.1. Allelic discrimination of the NOD2/CARD gene

Single nucleotide polymorphisms R702W, G908R, and (3020insC/L1007fsinsC) also known as SNP8, 12, and 13 of the NOD2 gene were determined by a Taqman protocol (performed by Heike Bremm, AG Holler, Internal Medicine III, University Hospital Regensburg), as previously described [33]. Alternatively, samples were analysed using a Sequenom MassARRAY iPLEX platform (performed by AG Rehli, Internal Medicine III,

University Hospital Regensburg). The assay consists of an initial locus-specific PCR reaction, followed by single base extension using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. Using Matrix Assisted Laser Desorption Ionization- Time-of-Flight (MALDI-TOF) mass spectrometry, the distinct mass of the extended primer identifies the SNP allele [100].

4.4. Biochemical methods

4.4.1. Protein isolation

For protein isolation, monocytes were isolated as described in section 4.1.1.
$2x10^6$ cells were seeded in 6-well plates and stimulated for 30 minutes. Afterwards, plates were transferred to ice, the cells harvested and centrifuged at 1300 rpm for 7 minutes at 4°C. Possible remaining cells in the 6-well plates were obtained by washing the wells with ice cold PBS. For later determination of protein phosphorylation 500 µl of freshly prepared pre-equilibration buffer (buffer B) which contains cytoplasmic extraction buffer (CEB) (also called buffer A in this setting) was added to the cell pellet whereas 500 µl of buffer was added to the culture plates to obtain any remaining cells. Cells were incubated in buffer for 4 min on ice and collected. Lysates were centrifuged and the supernatant carefully discarded. Subsequently, 60 µl of freshly prepared lysis buffer (buffer C) was added to the cell pellets and incubated for 10 min on ice. Afterwards, 60 µl of SDS buffer was added to the cell lysate and transferred to a 95°C heater for 10 min to denature the secondary protein structure. The obtained protein lysates were aliquoted, and stored at -80°C. The required buffers and solutions are presented in the tables bellow.

Table 4.5. Buffer A composition

Reagents	Final concentration	Final volume
Tris/HCl (pH 7,9)	10 mM	1 ml
KCl	60 mM	447 mg
EDTA	1 mM	37 mg
ddH_2O		ad 100 ml

Table 4.6. Buffer B composition

Reagents	Stock concentration	Final concentration	Final volume
EDTA	500 mM	1.5 mM	3 µl
Dithiothreitol	100 mM	1 mM	10 µl
EGTA	200 mM	1 mM	5 µl
β- Glycerophosphate	1 M	50 mM	50 µl
Sodium fluoride	1 M	50 Mm	50 µl
Sodium pyrophosphate	250 mM	25 Mm	100 µl
Sodium orthovanadate	200 mM	1 mM	5 µl
Leupeptin	1 mg/ml	2 µg/ml	2 µl
Pepstatin A	1 mg/ml	2 µg/ml	2 µl
Aprotinine	2 mg/ml	2 µg/ml	1 µl
Buffer A			772 µl
ddH_2O			ad 1000 µl

Table 4.7. Buffer C composition

Reagents	Stock concentration	Final concentration	Final volume
Nonidet P40	10%	0.4%	40 µl
Chymostatin	20 mg/ml	100 µg/ml	5 µl
Bestatin	5 mg/ml	10 µg/ml	2 µl
E64	3 mg/ml	3 µg/ml	1 µl
1,10-Phenanthrolin	0.1mg/ml	1 mM	1 µl
Buffer B			951 µl
ddH_2O			ad 1000 µl

Table 4.8. SDS sample buffer composition

Reagents	Final concentration	Final volume
Glycerine	20 %	10 ml
Tris buffer (pH 6.8)	125 mM	5 ml
Sodium dodecyl sulfate	4 %	2 g
2-Mercaptoethanol	10 %	5 ml
Bromophenol Blue	0.02 %	10 mg

4.4.2. SDS polyacrylamide gel electrophoresis

Protein samples obtained from section 4.4.1 were separated using a discontinuous gel system which is composed of a stacking and a separation gel mainly consisting of acrylamide (AA). Separation gel of 10% and stacking gel of 5% were prepared freshly as mentioned in table 4.9. In order to achieve complete polymerization, ammonimum persulfate (APS) was added acting as a free radical initiator and N, N, N', N'-tetramethylenedianime (TEMED) was added to catalyse the gel polymerization as mentioned in Table 4.10.

Table 4.9. SDS-PAGE stock solutions

Gel stock solution	Separation gel	Stacking gel
Final AA concentration	10%	5%
Stacking gel buffer	-	25 ml
Separating gel buffer	25 ml	-
SDS (10%)	1 ml	1 ml
Acrylamide (AA) (30%)	33 ml	16,7 ml
Millipore water	Adjust to 100 ml	Adjust?

Table 4.10-.SDS-PAGE gel mix solutions

	Separation gel	Stacking gel
Separating gel stock solution	6 ml	-
Stacking gel stock solution	-	2.5 ml
TEMED	6 µl	2.5 µl
APS (10%)	30 µl	20 µl

Table 4.11. Required buffers and solutions for SDS gel preparation

Solutions	Final concentration	Weight	Constituents
Separating gel buffer	1.5 M	90.83 g	Tris/HCl pH 8.8
Stacking gel buffer	0.5 M	30 g	Tris/HCl pH 6.8
Sodium dodecyl sulfate	10%	10 g	SDS
APS	10%	1 g	APS
Laemmli buffer (5X)	40 mM	15 g	Tris
Glycine	0.95 M	21 g	
SDS	0.5%	15 g	

To perform electrophoresis, the SDS gel was placed in the electrophoresis chamber and bathed in electrode buffer (1X). After carefully removing the comb, wells were rinsed with electrode buffer and loaded with equal volumes protein lysates (Chapter 4.4.1). Additionally, one well was loaded with 5 µl Kaleidoscope Prestained Standard (manufacturer) as molecular weight standard. Voltage of 80-100 V was applied and as soon as the samples reached the running gel, voltage was increased to 120-130 V. Electrophoresis was stopped at the time point when the lowest band of the protein marker reached the lower bottom of the gel.

4.4.3. Western blot analysis

Proteins separated by SDS-PAGE were transferred to a PVDF (Polyvinyl difluoride) membrane using a three-buffer semi-dry system. To transfer the proteins from the gel to the membrane, a PVDF membrane was cut into the size of the gel. The membrane was then soaked in 70% isopropanol and transferred to anode buffer B. Afterwards, three pieces of filter paper, of the size of the membrane, were soaked in anode buffer A, and placed on the semi-dry electro-blotting system. Three more filter papers were soaked in anode buffer B and placed on top of first three papers. Air bubbles were gently removed. With the help of forceps the membrane was placed over the filter paper. The SDS gel was carefully removed from the glass plate, immersed in buffer B for 10 s and placed on top of the membrane. Another 3 layers of filter paper soaked in buffer C were laid on top of the gel. The system was closed with the upper part of the semi-dry transfer cell (cathode) and transfer of the proteins onto the PVDF membrane was carried out at 11 V for 1 h at room temperature (RT).

In a second step, the proteins on the membrane were subjected to immunostaining. Each step was performed with shaking for maximum accessibility of reagents. To minimize non-specific binding, the membrane was incubated in blocking buffer over night at 4°C. Afterwards, the membrane was swirled in a dilution of primary antibody in blocking buffer (Chapter 3.8.1) for 1.5 h at RT. Then, the membrane was washed three times in Tris-buffered saline and Tween 20 (TBST) buffer for 10 minutes, before incubation with a dilution of a secondary antibody in blocking buffer (Chapter 3.8.1), specific to the isotype of the primary antibody for 1 hour at RT. The membrane was washed again three times in TBST buffer for 10 minutes, and proteins were visualized on an imaging film using a luminol-based enhanced chemiluminescence (ECL) solution (Table 4.10).

All the proteins of interest were normalized to the reference gene, β-actin, meaning that the previous antibody had to be stripped off. For this, membrane was washed three times for 10 min followed by stripping off the old signal with Reblot solution (1:1 diluted; manufacturer) for 15 min at RT. The membrane was then washed three times and

preceded with blocking, washing, and primary and secondary antibody procedure. Stripping was performed every time when more than one antibody was to be bound on the same membrane and these antibodies are isolated from the same species. Images were processed and analysed using Image Lab software.

Table 4.12. Western blot buffers

Buffers	Components
Buffer A	0.3 M (36.3 g) Tris/HCl (pH 10.4) 20% (200 ml) Methanol Add Millipore water to 1000 ml
Buffer B	25 mM (3.03 g) Tris/HCl (pH 10.4) 20% (200 ml) Methanol Add Millipore water to 1000 ml
Buffer C	4 mM (5.2 g) Caproic acid (pH 7.6) 20% (200 ml) Methanol Add Millipore water to 1000 ml
TBS buffer (2X)	20 mM (9.16 g) Tris/HCl (pH 7.4) 150 mM (35.1 g) NaCl Add Millipore water to 2000 ml
TBST buffer	1X (500 ml) TBS (2X) 0.1% (1 ml) Tween 20 Add Millipore water to 1000 ml
Blocking buffer	5% (5 g) Non-fat dry milk Add 100 ml TBST buffer

Table 4.13. Composition of ECL solutions

Solutions	Components
ECL solution A	250 mM (100 mg) Luminol Add 2 ml of Millipore water
ECL solution B	90 mM (14.8 mg) *p*-Coumaric acid Add 1 ml DMSO
ECL mix	2 ml ECL solution A 0.89 ml ECL solution B 20 ml Tris/HCl (pH 8.5, 1 M) Add 200 ml ddH_2O Stored away from sunlight.
ECL working solution	5 ml ECL mix + 1.5 µl 30% H_2O_2

4.5. Mouse experiments

All mouse experiments were conducted according to the regulations of the government of Upper Palatinate, Regensburg, Germany and in cooperation with AG Hoffmann/Edinger (Internal Medicine III, University Hospital Regensburg, Germany). Female BALB/c mice and C57BL/6 mice at the age of 3 weeks were purchased from Charles River Laboratories. CD-1 mice were gently provided by the Central Animal Facility, University Hospital Regensburg.

4.5.1. Vitamin D_3 deficient *versus* sufficient mouse model

In order to generate a model differing in serum Vitamin D_3 levels, BALB/c or CD-1 mice at the age of 3 weeks were fed with special Vitamin D_3-deficient (<100 U/Kg) or sufficient (1500 U/Kg) diets (Ssniff GmbH). Serum 25(OH)D_3 levels were measured by means of liquid chromatography high-resolution tandem mass spectrometry by the Clinical Chemistry Department, University Hospital Regensburg.

4.5.1.1. Preparation of murine tissue for flow cytometry analysis

For flow cytometry analyses, tissues of mice had to be processed to single-cell suspensions. In order to maintain cell viability, tissues dissected from mice were collected in ice-cold PBS and tissue preparation was performed on ice.

Spleens were processed to single-cell suspensions by scratching out cells with curved forceps in a petri dish containing medium. Afterwards, they were filtered via 100 µm-cell strainers and centrifuged. Erythrocytes in single-cell suspensions were lysed with 2 ml ACK (Ammonium-Chloride-Potassium) lysis buffer (0.155 M NH_4Cl, 0.1 M $KHCO_3$ and 0.1 mM EDTA, diluted 1:6 with H_2O) for 3 minutes at room temperature. The reaction was stopped by adding 10 ml medium and cells were centrifuged. Cell pellets were resuspended in 1-50 ml medium (volume dependent on pellet size) and filtered via 100 µm-cell strainers. After counting the cells with a CASY cell counter, 0.5-1 x 10^6 cells were stained for flow cytometry analysis.

Bone marrow was collected flashing the cells out of the bone with medium with the help of a syringe. Afterwards, the collected cells were pressed into a 100 µm-cell strainer, centrifuged and the erythrocytes lysed like described above.

4.5.1.2. Preparation of blood samples

Blood of mice was collected from the tail vein in heparin tubes. 2-3 drops of blood were lysed with 1 ml ACK lysis buffer (Chapter 4.4.2.2) for 5 minutes at room temperature. The reaction was stopped by adding 2 ml flow cytometry buffer (PBS containing 2% FCS). After centrifugation, supernatants were carefully aspired, leaving ~50 µl to avoid

disturbing the pellet and ACK lysis was repeated until the pellet was colourless. At last, the pellet was resuspended in 1 ml of flow cytometry buffer for subsequent flow cytometry analysis.

4.6. Microbiota analysis

In recent years, significant advances in “deep-sequencing technologies” made possible the identification and characterization of the intestinal microbial content. The most common tool used for taxonomic characterization of microbial communities is the 16S ribosomal DNA sequencing. This gene has both highly conserved and variable regions. By amplifying regions of the gene using primers against the conserved regions, one can generate a population of bar-coded sequences that contain the region internal to those primers, which is highly variable. These regions can then be sequenced and classified based on the similarity to a reference database [51, 101].

For the microbiota analysis, stool samples of the mice were collected and frozen at -20°C until analysis. The gut microbiota composition of the mice was analyzed by means of 16 rDNA PCR analysis (Dr. Andreas Hiergeist, AG Gessner, Institute of Microbiology and Hygiene, University Hospital Regensburg) as previously described [102].

4.7. Statistical analysis

Results represent the median or mean and standard error of the mean (s.e.m.). Comparison between two groups was performed using Mann-Whitney U test or with Wilcoxon signed rank test (non-normal distributed data). When analysing more than two groups, non-normal distributed data was analysed with Kruskal-Wallis test followed by Dunns post-test. In case of normal- distributed data one-way ANOVA was used followed by Tuckey´s post-test. *P*-values of < 0.05 were considered statistically significant. All calculations were performed using the GraphPad Prism software.

5. Results

5.1. Impact of NOD2 polymorphisms on the immune cell composition in peripheral blood of healthy donors

NOD2/CARD15, the intracellular pathogen-recognition receptor recognizing bacterial muramyl dipeptide (MDP), has gained substantial interest, as single nucleotide polymorphisms (SNPs) of this receptor have been identified as a risk factor for Crohn´s disease and Graft-versus-Host disease (GvHD) [33, 38] . These data suggest that MDP sensing by hematopoietic cells is of crucial importance for the immune homeostasis in target organs of GvHD, especially in the gastrointestinal tract and/or skin.

We were interested whether differences in the blood cell composition would be present in individuals carrying the respective SNPs (8, 12 and 13) in the NOD2 receptor.

These mutations affect the C-terminal portion of the NOD protein. While SNP8 and SNP12 lead to amino acid substitutions, SNP13 represents a frameshift insertion. Both SNP12 and 13 mutations are located at the leucin-rich repeat (LRR), the region for ligand recognition, while the SNP8 mutation is located in the region between NOD and in close proximity to LRR [45].

Blood samples from healthy donors without NOD2 mutation (WT) and donors carrying NOD2 SNP8, 12 and/or 13 NOD2 heterozygous mutations were collected into heparin containing tubes; the PMNCs were isolated by ficoll density gradient centrifugation and cryopreserved for future analysis.

Seven-color flow cytometry was performed using standard protocols for cell surface staining to establish the frequencies of different cell populations. The percentage of T cells was determined by CD3 staining. B cells were determined via the percentage of $CD19^+$ cells, myeloid cells by CD33 staining and NK cells by excluding the $CD3^+$ cells and gating on $CD16^+/CD56^{dim}$ cells.

As observed in figure 5.1, no differences were found regarding the percentages of T and B cells. However, a significant lower percentage of $CD33^+$ myeloid cells was found in individuals bearing any of the NOD2 SNPs. Regarding NK cells, we observed a trend towards an increase in this population in SNP donors. Next, a more detailed analysis of the populations was performed, and specific cell sub-populations were analyzed.

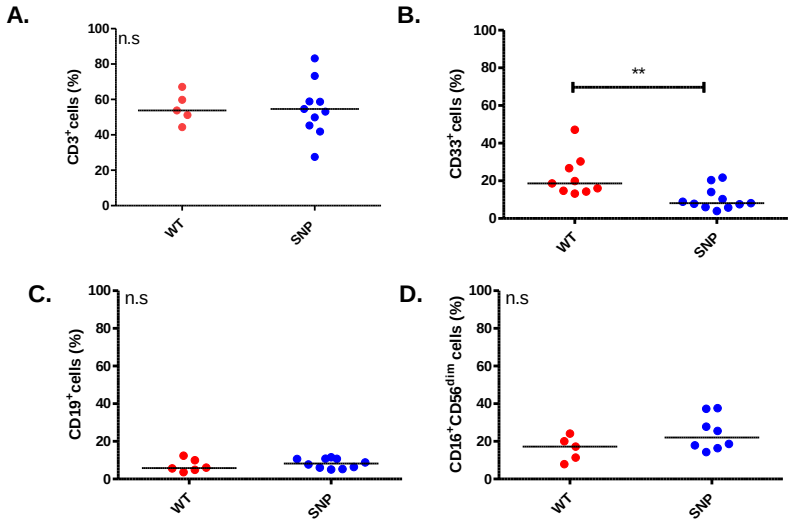

Figure 5.1. Impact of NOD2 SNP mutations on the blood composition of healthy donors. Percentage of $CD3^+$ T cells (A), $CD33^+$ myeloid cells (B), $CD19^+$ B cells (C) and $CD16^+CD56^{dim}$ NK cells (D) among living $DAPI^-$ leukocytes. Each symbol represents an individual donor. Statistical analysis was performed with Mann Whitney U test. (** $p \leq 0.01$, n.s.: not significant).

For the characterization of T lymphocyte populations, cells were stained for CD4, the marker for T helper cells, CD8, the marker for cytotoxic T cells, and CD25, a marker for T cell activation, also used for the identification of regulatory T cells (Tregs), since Tregs express constitutively high levels of CD25.

In line with the preserved amount of $CD3^+$ cells, no differences were found regarding the amount of specific T cell subtypes, as a similar percentage of $CD4^+$,$CD25^+$ and $CD8^+$ cells was observed in both WT and SNP donors.

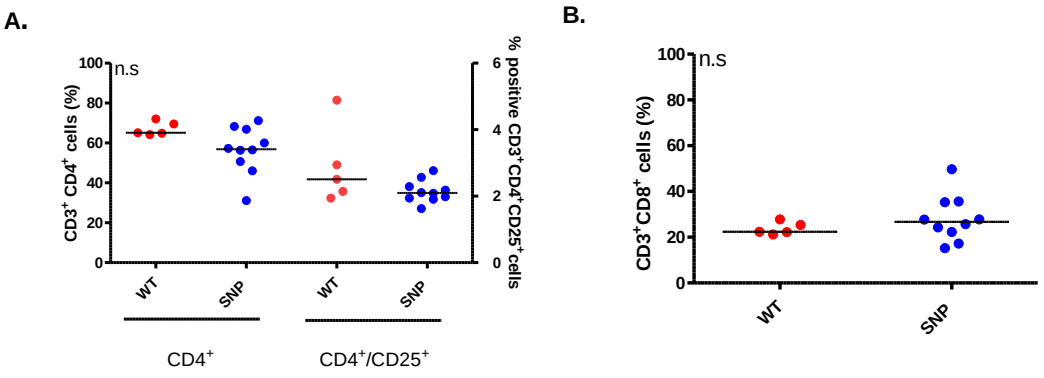

Figure 5.2. Amount of $CD4^+$, and $CD4^+CD25$ (A) and $CD8^+$ (B) T cells among living $CD3^+$ cells in the blood of WT and donors with NOD2 variants. Each symbol represents an individual donor and horizontal lines indicate the median. Statistical analysis was performed with Mann Whitney test. n.s.: not significant)

The dendritic cell populations present in blood were also analysed (Figure 5.3). The amount of plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) were determined by staining the cells with a lineage cocktail (CD3, CD14, CD19, CD20, CD56) to exclude T cells, B cells, monocytes and NK cells. After gating on HLA-DR+ cells, CD11c and CD123 were used to further discriminate the DC populations. mDCs were identified as lineage negative cells with high expression of CD11c and no expression of CD123, whereas pDCs express CD123 and have no CD11c expression.

We observed a significantly reduced amount of mDCs ($CD11c^+$, $CD123^-$) in the SNP donors compared with WT. We also found a trend towards an increase in pDC frequency in SNP donors ($CD11c^-$, $CD123^+$) compared to WT, however, this effect was not statistically significant.

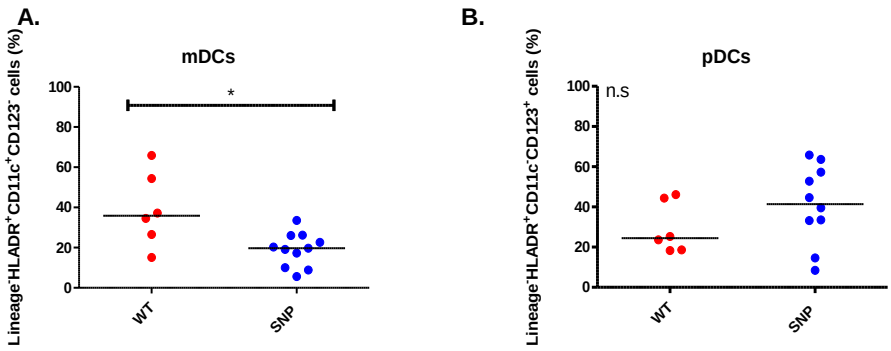

Figure 5.3. Amount of myeloid (A) and plasmacytoid (B) dendritic cells among living lineage negative HLA-DR$^+$ cells in the blood of WT, SNP8, 12 and 13 healthy donors. Each symbol represents an individual donor and horizontal lines indicate the median. Statistical analysis was performed with Mann Whitney U test (** $p \leq 0.01$, n.s.: not significant).

As the number of $CD33^+$ cells was significantly reduced, we were interested whether $CD33^+$ subpopulations were influenced by the NOD2 SNPs. Therefore, we excluded cells positive for CD3, CD56 or CD19 and investigated CD33 expression as well as CD14 and CD16, markers characterizing subpopulations of monocytes. As shown in figure 5.4 A, we identified two $CD33^+$ populations differing in the amount of CD33 expression ("low" – figure 5.4.B, "high" - figure 5.4.C). SNP donors showed a reduction in the CD33 "high" population compared to WT (Figure 5.4 C). Further analysis of this CD33 "high" population demonstrated an increase in CD16 expression on monocytes (Figure 5.4 D). Interestingly, donors with SNP8 showed comparable expression levels to WT donors, while donors with SNP12 or 13 showed increased expression levels.

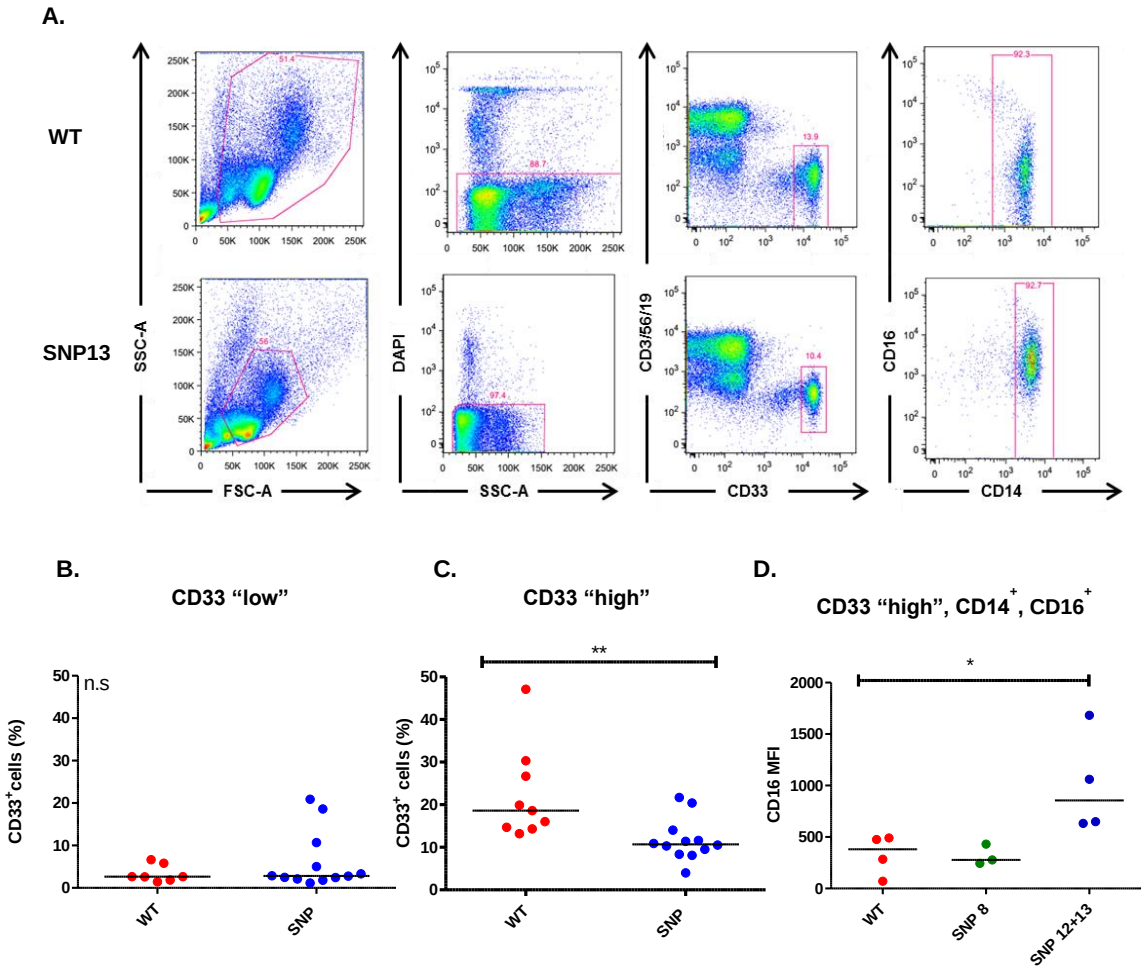

Figure 5.4. Amount of CD33^{+} cells among living leukocytes in the blood of WT, SNP8, 12 and 13 healthy donors. (A) gating strategy and representative image of a WT and SNP13 donor; (B)- amount of CD33 "high"; (C) Amount of CD33 "low"; (D) mean fluorescence intensity (MFI) of CD16 expression on monocytes, gated on CD33 "high". Each symbol represents an individual donor and horizontal lines indicate the median. Statistical analysis was performed with Mann-Whitney U test (B and C) and Kruskal–Wallis test (D) (* $p \leq 0.05$, ** $p \leq 0.01$, n.s.: not significant).

5.1.1. Impact of NOD2$^{-/-}$ on immune cell composition in mice

The number of donors carrying SNPs in NOD2 is rather limited. In the duration of the present work, 10% of the donors were carriers of SNP8, 4% of SNP12 and 5% were SNP13 carriers. Therefore, we investigated whether a mouse model lacking NOD2 would be an appropriate model reflecting the situation in humans carrying NOD2 SNPs. However, this model did not reflect the human findings, as the differences found in the myeloid compartment were not observed in mice lacking NOD2 expression (Figure 5.5). For this reason, all further experiments were performed with human samples.

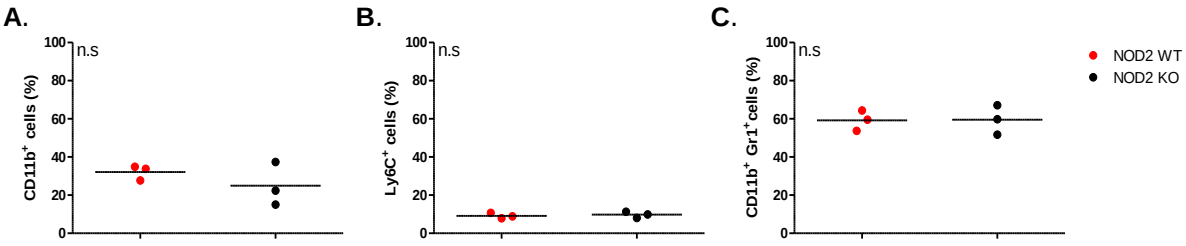

Figure 5.5. Impact of NOD2 expression on myeloid blood cells of WT and NOD2$^{-/-}$ mice. Amount of total myeloid cells (CD11b^{+}) **(A)** ,total amount of monocytes (Ly6C^{+}) **(B)** and amount of CD11b^{+}Gr1^{+}myeloid-derived suppressor cells (MDSC) **(C)** in the blood of WT and NOD2$^{-/-}$ mice. Each symbol represents an individual donor and horizontal lines indicate the median. Statistical analysis was performed with Mann-Whitney U test (n.s.: not significant).

5.2. Impact of blood sampling and cell isolation method on the cell phenotype

In the previous experiments, blood collected with heparin tubes followed by ficoll separation was used. Due to the low yield of cells, the number of analysis was limited. As we observed a significant difference in donors with NOD2 SNPs regarding the myeloid compartment, we decided to study this cell population in more detail. Consequently, to obtain larger cell numbers, leukapheresis volunteers were screened for NOD2 polymorphisms. Surprisingly, we could not reproduce the increased CD16 expression on monocytes with NOD2 SNPs using PMNCs from leukapheresis products. In fact, a trend towards an even lower expression of CD16 in donors carrying the SNP12 or 13 was observed.

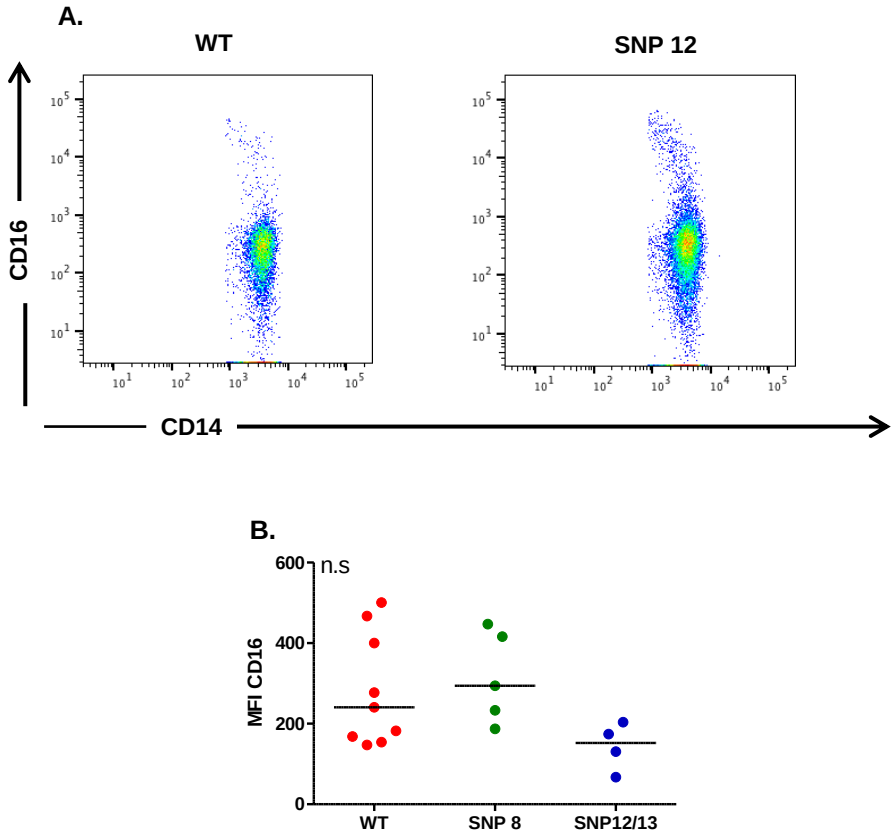

Figure 5.6. Median fluorescence intensity (MFI) of CD14^{+}16^{+} monocytes among living CD33^{+} cells in the blood of WT, SNP8, 12 and 13 healthy donors. MNCs were collected from leukapheresis products followed by ficoll separation. Each symbol represents an individual donor and horizontal lines indicate the median. Statistical analysis was performed with Kruskal–Wallis test (n.s.: not significant).

Based on these conflicting data, we wondered whether the isolation method has an impact on CD16 expression. In the first set of experiments cells were collected using heparin as anti-coagulant agent; in the second set of experiments cells were obtained via leukapheresis products which uses citrate as anti-coagulant. In order to investigate a possible effect of the anti-coagulant on the monocyte phenotype, cells from the same donor were collected using different methods. Blood was withdrawn using tubes coated with different anti-coagulants (heparin, citrate, hirudin) before leukapheresis (always with citrate) from each donor. All samples were further separated by ficoll to isolate MNCs, which were stained for CD16 expression. Moreover, MNCs from the leukapheresis products were further separated by elutriation to obtain pure monocytes and also stained for CD16 expression, to control whether the elutriation process itself has an impact on the cell phenotype.

As shown in figure 5.7, the collection method had a strong impact on the cell phenotype, as we observed a significant decrease in CD16 expression in cells collected by

leukapheresis. However, citrate alone had no significant impact on CD16 expression, showing that the decreased expression observed in leukapheresis products is possibly due to the preparation procedure itself but not due to the use of citrate. Elutriated monocytes also showed a lower CD16 expression, comparable to mononuclear cells from leukapheresis products before the elutriation, demonstrating that the elutriation procedure did not further modulate CD16 expression.

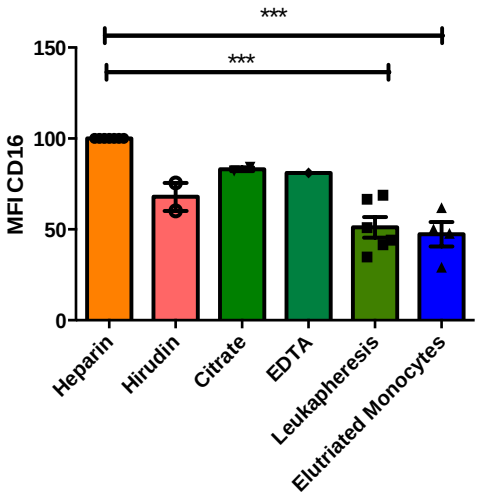

Figure 5.7. Impact of the blood collection method on CD16 expression. Blood was drawn either in the presence of heparin (n=6), hirudin (n=2), citrate (n=2) or EDTA (n=1). The same donor donated blood cells for the leukapheresis product. All blood samples were separated by ficoll. Elutriated monocytes separated from leukapheresis products are also shown (n=4).Shown is the median fluorescence intensity (MFI) of CD16. Each dot represents one individual donor. Statistical analysis was performed with Kruskal–Wallis test (** $p \leq 0.01$, *** $p \leq 0.001$).

5.2.1. CD16 downregulation: Importance of the recognized epitope

The FcγRIII (CD16) is a low-affinity receptor for monomeric IgG. This glycoprotein of 50 to 70 kD is expressed on neutrophils, natural killer cells, eosinophils, macrophages and monocytes. The CD16 expressed on natural killer cells and macrophages (CD16A) has a classical polypeptide membrane anchor, whereas the neutrophil CD16 (CD16B) is glycosyl-phosphatidyl inositol (GPI)-anchored [103].

The antibody used in the previous experiments was produced by the hybridoma cell clone 3G8, which should recognize both A and B isoforms of CD16. Next, we aimed to investigate if the observed difference in CD16 expression between the different preparation procedures was epitope-specific. Therefore, in addition to the previously used 3G8 antibody, also two other antibodies directed against both CD16 isoforms (A and B) from different clones, B73.1 and VEP13 were used. It is known that the epitope recognized by clone B73.1 is in the first membrane distal Ig-like domain of the CD16 molecule, which is different from that of clones 3G8 and VEP13.

Donor-matched blood from healthy volunteers was drawn using heparin tubes and erythrocytes were lysed using ACK lysis buffer. Alternatively, the same donors' MNCs were obtained from ficoll separation after leukapheresis, as already described.

Results demonstrated that the downregulation of CD16 expression in monocytes collected by leukapheresis was significant when stained with the 3G8 clone and the VEP13 clone but not when CD16 was measured using the B73.1 clone (Figure 5.8). Therefore, it seems that the observed decreased expression of CD16 detected with 3G8 and also with VEP13 represents a downregulation of specific epitopes whereas other epitopes (detected by B73.1) are preserved.

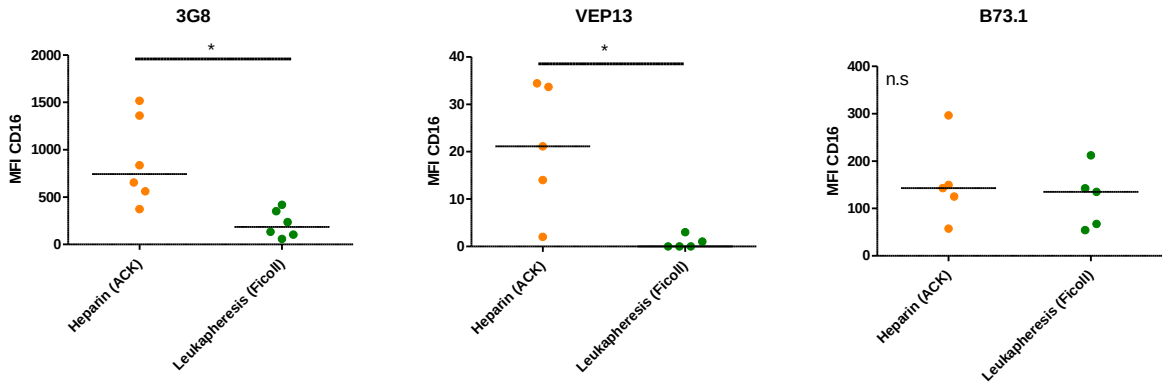

Figure 5.8. Relative CD16 expression on monocytes using different antibody clones. Cells were collected either with heparin tubes (orange) or by leukapheresis, using citrate as an anti-coagulant (green) and stained with different CD 16 antibodies. Shown is the CD16 MFI. Each dot represents one individual donor and horizontal lines indicate the median. Statistical analysis was performed with Wilcoxon signed rank test (* $p \leq 0.05$, n.s.: not significant).

Interestingly, Passacquale et al. reported of an interaction between platelets and monocytes which they found to up-regulate CD16 expression in monocytes [104]. These monocyte-platelet aggregates are calcium-dependent and therefore calcium chelators such as citrate or EDTA could lead to a disruption of these aggregates, thereby reducing the amount of $CD16^+$ monocytes. On the other hand, heparin as anti-coagulant could preserve the interaction of platelets and monocytes.

5.2.2. Effect of platelets and TGF-β on human monocytes

Because platelets are a major source of TGB-β in circulation [105], it was hypothesized that the up-regulation of CD16 in monocytes would also occur in monocytes treated with TGF-β.

To prove that platelets and TGF-β are capable to induce the expression of CD16, monocytes were isolated by elutriation and co-cultured with platelets or with TGF-β in the presence or absence of MDP (the natural ligand of NOD2) for 24 hours. After this period, cells were harvested and stained for CD33, CD14, CD16 (3G8 clone) and CD62P, a

marker for activated platelets. Co-expression of CD62P and CD14 demonstrated monocyte-platelet aggregates (Figure 5.9 A). Although a significant up-regulation was not detected, we observed a clear trend towards an increase of $CD16^+$ monocytes in the presence of platelets and TGF-β, especially upon MDP treatment (Figure 5.9 B).

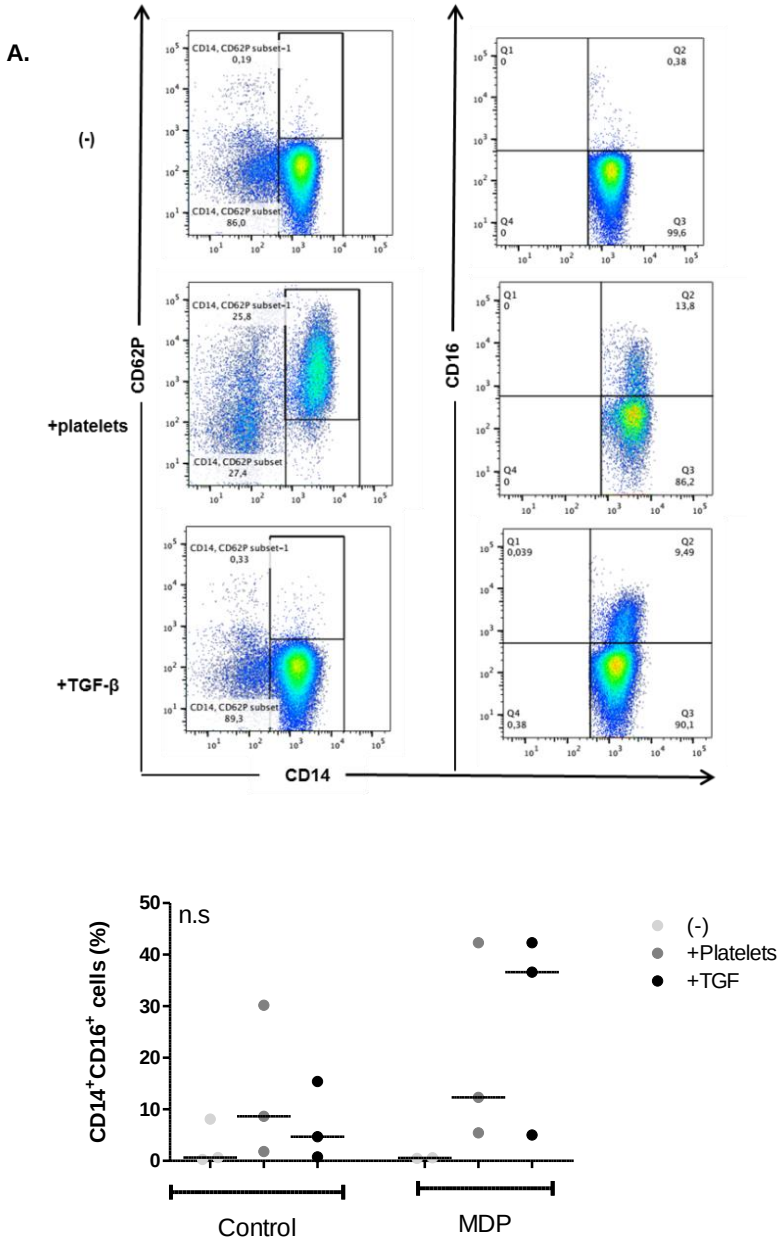

Figure 5.9. Percentage of $CD16^+$ WT monocytes co-cultured with platelets or TGF-β. 2 $x10^6$ Monocytes were cultured in 2 ml RPMI media supplemented with 2% AB serum. After 24 hours, the cells were harvested, washed and stained with fluorochrome-conjugated monoclonal antibodies **(A)**. Shown is the median of $CD14^+$ $CD16^+$ cells. Each dot represents an individual donor. (n=3) **(B)** Statistical analysis was performed with Kruskal–Wallis test (n.s.: not significant).

Next, CD16 expression was investigated in monocytes from NOD2 SNP-bearing donors under the same conditions. For this purpose, frozen cells from WT, SNP8, 12 and 13 donors were cultured for 24 hours with or without TGF-β, LPS or MDP. Cell staining before starting the culture (day 0) revealed a low level of CD16 expression (Figure 5.10). Donors carrying a SNP12 or SNP13 (SNP12/13) had a slightly higher percentage of $CD16^+$ cells, compared to WT and SNP8 donors after 24 hours incubation without any treatment. MDP stimulation had no impact on CD16. TGF-β stimulation was able to up-regulate CD16 in all monocytes. The strongest effect of TGF, however, was observed in monocytes from SNP12/13 donors. Upon LPS treatment, we observed a lower expression of CD16 in donors with NOD2 SNP12/13 (Figure 5.10).

These results indicate that the higher CD16 expression of donors with NOD2 SNP12/13 found in mononuclear cells collected with heparin tubes but not in cells from leukapheresis products can be partially recovered after culturing the cells for 24 hours *in vitro*. Furthermore, TGF-β up-regulates CD16 independently of the NOD2 status. Nevertheless donors with NOD2 SNP12/13 were more susceptible to this effect since they exhibited a significantly higher expression of CD16. Interestingly, LPS counteracted the up-regulation of CD16 independently of the NOD2 status. However, more donors have to be included in the analysis to confirm the observed effects.

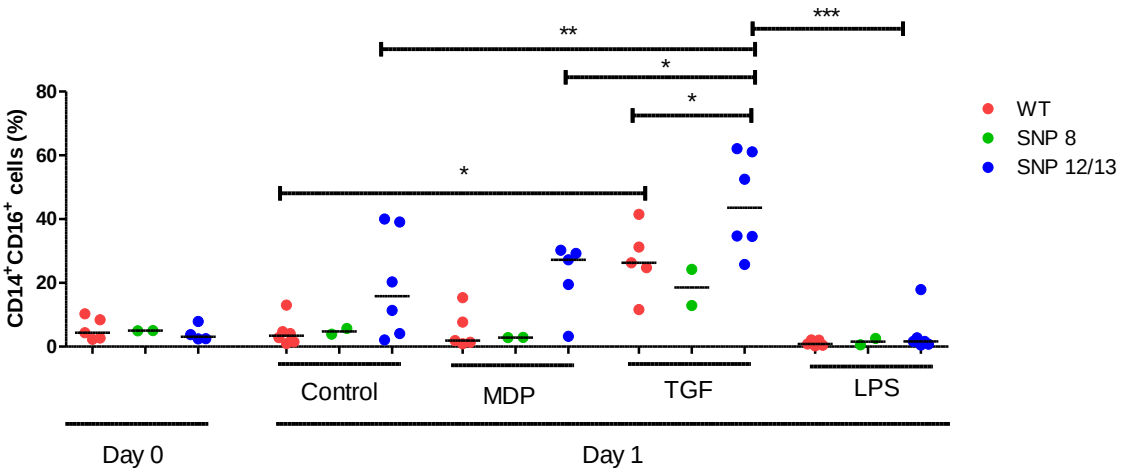

Figure 5.10. Percentage of $CD14^+CD16^+$monocytes stimulated with or without MDP, TGF-β or LPS. 2 x10^6 frozen monocytes were cultured in 2 ml RPMI medium supplemented with 2% AB serum and stimulated with the indicated substances. After 24 hours, cells were harvested, washed and stained with fluorochrome-conjugated monoclonal antibodies. Shown is the median of $CD16^+$ positive cells. Each dot represents an individual donor. Statistical analysis was performed with Kruskal-Wallis test. Due to the low number of SNP8 donors, they were not included in statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.)

As a control we analysed whether other typical monocyte antigens such as CD14 (Figure 5.11) are also altered in cultured monocytes under the different treatments. Here no significant differences were observed between the treatments independent of the NOD2

status, although a trend could be observed towards a down-regulation of CD14 upon LPS stimulation.

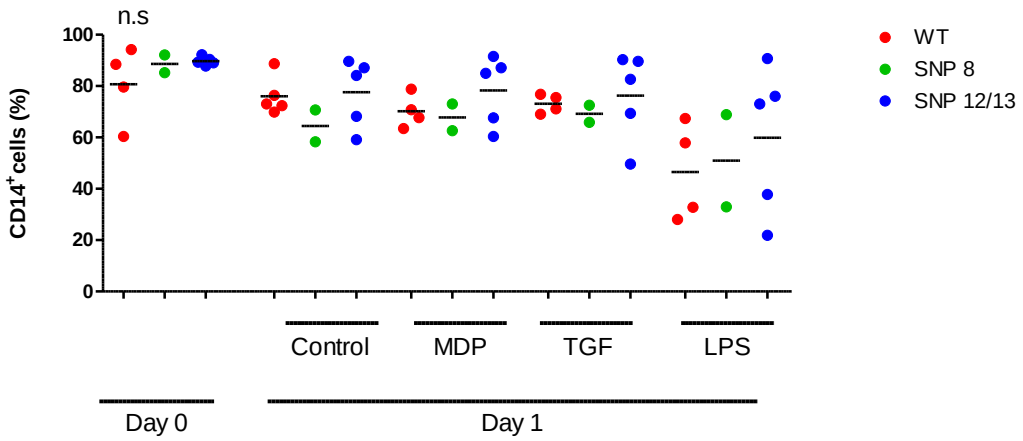

Figure 5.11. CD14 expression on monocytes stimulated with or without MDP, TGF-β or LPS. 2 x10^6 frozen monocytes were cultured in 2 ml RPMI medium supplemented with 2% AB serum and stimulated with the indicated substances. After 24 hours, cells were harvested, washed and stained with fluorochrome-conjugated monoclonal antibodies. Each dot represents an individual donor. Shown is the median of $CD14^+$ positive cells. Statistical analysis was performed with Kruskal-Wallis test (n.s.: not significant). Due to the low number of SNP8 donors, they were not included for statistical analysis.

In addition, HLA-DR expression was analysed on monocytes. HLA-DR is an important surface marker regarding antigen presentation. Monocyte culture or MDP treatment did not influence HLA-DR expression on monocytes independent of the NOD2 status (Figure 5.12).

TGF-β down-regulated HLA-DR expression however this effect was only significant for donors with NOD2 SNPs 12/13.

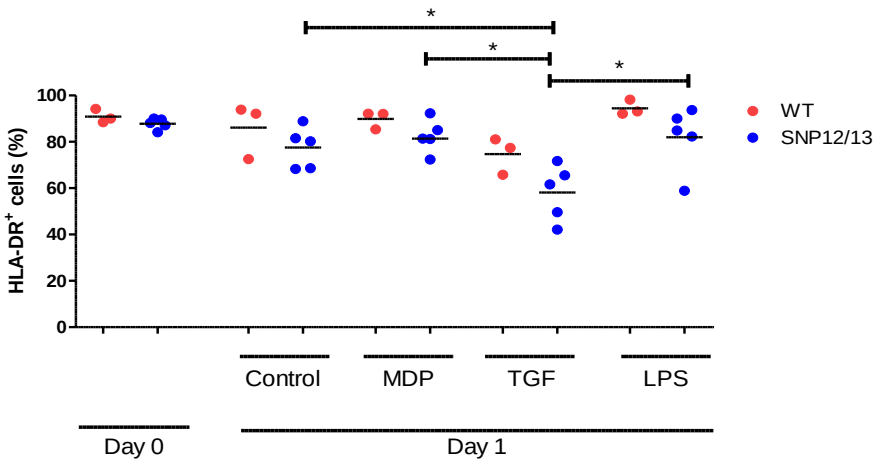

Figure 5.12. Percentage of $HLA\text{-}DR^+$ monocytes stimulated with or without MDP, TGF-β or LPS. 2x10^6 frozen monocytes were cultured in 2 ml RPMI medium supplemented with 2% AB serum and stimulated with the indicated substances. After 24 hours, the cells were harvested, washed and stained with fluorochrome-conjugated monoclonal antibodies. Each dot represents an individual donor. Shown is the median of $HLA\text{-}DR^+$ positive cells. Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$).

In summary, the phenotype with regard to surface markers of monocytes from donors with NOD2 SNP12 or 13 was altered. Cells expressed similar amounts of CD14 but increased levels of CD16. Furthermore, monocytes with NOD2 SNP12 or 13 were more susceptible to TGF-β treatment, which up-regulated CD16 but down-regulated HLA-DR expression compared to donors with WT NOD2. Next, the functional impact of the NOD2 status was determined in monocytes by means of analysis of cytokine expression.

5.2.2.1. Cytokine profile of monocytes with different NOD2 variants

The cytokine secretion was analysed by means of ELISA in supernatants of the previous cultures used for surface marker analyses (Figures 5.10-5.12). The levels of IL1-β, IL-6 and IL-8 and TNF were determined.

We observed that WT and NOD2 SNP donors produced similar amounts of IL-6 independent of the stimulus. Although not significant, in WT individuals IL-6 production was diminished upon incubation with the combination of LPS and MDP compared to incubation with LPS alone. This effect, however, was not observed in the NOD2 SNP donors. Furthermore, non-stimulated monocytes (control) showed increased IL-6 production compared with WT (Figure 5.13 A).

Although IL-8 secretion was slightly decreased in donors carrying the NOD2 SNPs in all treatments, statistical significance was only reached in the presence of MDP in donors with NOD2 SNP12 or 13 compared to WT donors. This is in line with some reports that already described that IL-8 levels are decreased in patients with NOD2 SNPs [106].

TNF secretion (Figure 5.13C) was not significantly altered between WT and NOD2 SNP donors, with LPS treatment or LPS and MDP combination being able to up-regulate cytokine production independent of the NOD2 status.

Although a trend towards a decrease in IL-1β secretion is observed in donors bearing the NOD12/13 mutation in control, MDP and TGF-β treatment this was not significant. Upon LPS or LPS in combination with MDP treatment, a significant up-regulation of IL1-β was observed, independent of the NOD2 status (Figure 5.13D)

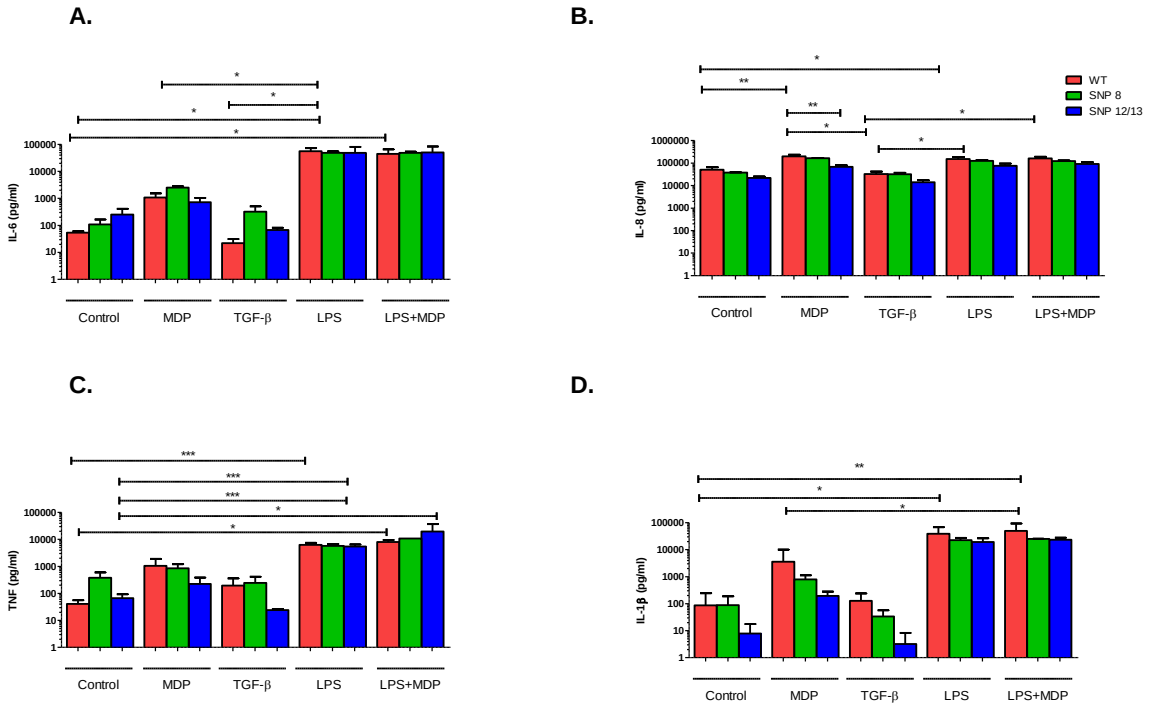

Figure 5.13. Amount of IL-6 (A) , IL-8 (B), TNF (C) and IL1-β (D) secretion in monocytes stimulated with or without MDP, TGF-β and LPS or LPS+MDP. Statistical analysis was performed with Kruskal-Wallis test (*$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). WTn= 5, SNP8 n=2, SNP12/13 n=6 Due to the low number of SNP8 donors, they were not included for statistical analysis.

5.2.2.2. Regulation of IκBβ and phospho p38 expression in monocytes with different NOD2 variants

It is known that NOD2 activation triggers different signalling pathways including the NF-κB pathway. Upon stimulation with pro-inflammatory cytokines such as TNF, IL-1β or bacterial stimuli, IκBα/β is phosphorylated, polyubiquitinated, then degraded by the 26S proteasome, allowing NF-κB to translocate to the nucleus and activate its target genes [107]. In order to investigate if monocytes from donors with NOD2 WT or NOD2 SNPs exhibit differences regarding this pathway, IκB protein was analysed by western blot. Both MDP and LPS were able to reduce IkBb expression in the WT donor. Interestingly, we found an almost undetectable level of IκB expression in non-stimulated cells of the donor with a combined SNP8+13 mutations compared to the WT donor suggesting a constitutively active NF-κB pathway. In line with this finding, this donor had a constitutive high production of IL-6 (Figure 5.14 C). The analysed donor harbouring the SNP13 alone revealed a similar behaviour to the WT, possibly indicating that the presence of 2 different mutations (SNP8+13) represents a special phenotype with stronger consequences in terms of cell signalling. These results have to be confirmed with more donors harbouring NOD2 SNPs.

The activation of phospho-p38 MAP Kinase was also analysed in WT, SNP8+13 and SNP13 (Figure 5.14C). A high variance was detected in the two analysed NOD2 WT in the control group. One donor showed only p38 activation upon stimulation whereas the other presented a strong activation of p38 in the control. The analysed SNP8+13 appears to activate this pathway with all the applied stimuli, whereas for the SNP13 donor no significant differences were detected regarding p38 activation.

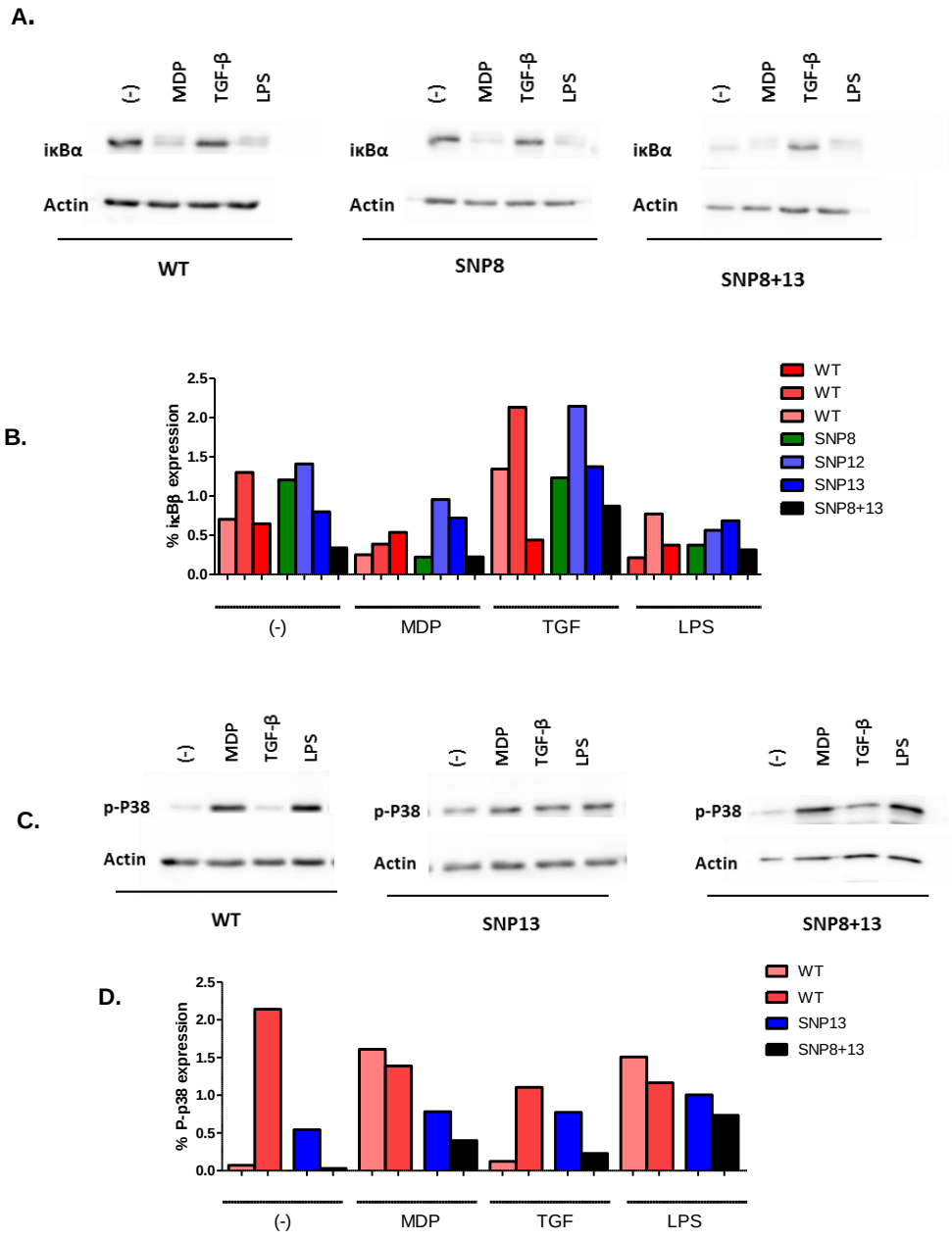

Figure 5.14. Effect of NOD2 mutations on iκBβ and phospho p38 expression. Monocytes were stimulated for 30 minutes with the indicated stimuli. Phospho-protein lysates were collected and western blot was performed to analyse iκBβ expression (A and B) Representative blot is shown on A and densiometric analysis in shown on B. On C is shown representative blots for P-p38 and on D the densiometric analysis WT n≥2, SNP8 n=1 SNP13+8 n=1; SNP13 n=1.

5.3. Impact of NOD2 on monocyte-derived macrophages

Macrophages (and their precursors, monocytes) are the 'big eaters' of the immune system. These cells are present virtually in all tissues. They differentiate from circulating blood monocytes which migrate into tissues in the steady state or in response to inflammation. Macrophages are remarkably plastic and can change their phenotype depending on the environmental signals they receive. Through their ability to clear pathogens and instruct other immune cells, these cells have a central role in protecting the host but can also contribute to the pathogenesis of inflammatory diseases [13].

It is generally accepted that $CD14^{++}$ classical monocytes leave the bone marrow and differentiate into $CD14^{++}CD16^{+}$ intermediate monocytes and sequentially to $CD14^{+}CD16^{++}$ non-classical monocytes in the peripheral blood circulation [108].

Due to the observation of an increased amount of $CD14^{++}CD16^{+}$ monocytes in donors with NOD2 SNP12 and SNP13, we hypothesised that the differentiation of macrophages and/or dendritic cells could be impaired, leading to the accumulation of “mature” monocytes in the blood circulation. Since macrophages normally express high levels of CD16 as a differentiation marker, this cell type appear to be a promising cell population to study the CD16 regulation in NOD2 mutated donors. For that reason, monocytes were cultured from donors with NOD2 WT and SNP13 in teflon bags with AB-serum for 7 days. After this period cells were harvested and stained for macrophage markers. Data analysis demonstrated no significant impact of the NOD2 SNP13 mutation on macrophage maturation indicating that the maturation of macrophages is not disturbed by the presence of NOD2 SNP13. Nevertheless, a decreased expression in CD16 was detected on the analysed NOD2 SNP13 donor. Since a great variation between donors sharing the same NOD2 genotype was identified, no significant conclusion can be taken from these experiments. The number of viable cells (Figure 5.15 B) was the same in the analysed WT and SNP donors. Nonetheless, data should be confirmed with other donors carrying the NOD2 SNP12/13 mutation.

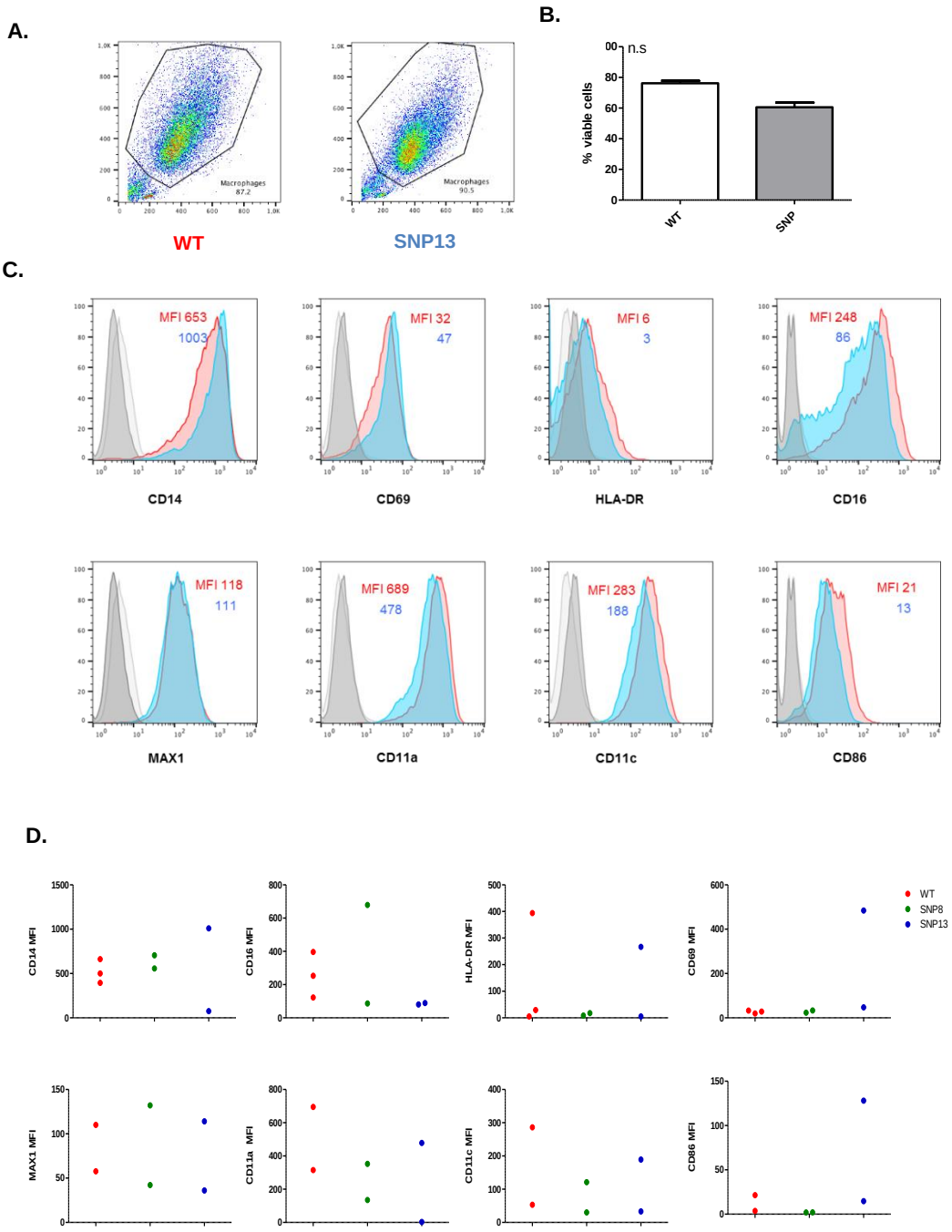

Figure 5.15. Expression of maturation markers in monocyte-derived macrophages from donors with NOD2 WT and NOD2 variants. Macrophages were cultured as mentioned before. Cells were harvested, washed, and stained with the indicated fluorochrome-conjugated monoclonal antibodies. Samples were analysed by flow cytometry. Representative FSC and SSC **(A)** cell viability **(B)** representative histograms (C) summary of the preformed experiments **(D).** Shown is the median fluorescence intensity (MFI) (isotype subtracted). Cell viability was determined by a cell analyzer (CASY system).

5.4. Impact of NOD2 on dendritic cell differentiation cultures

Due to the observed reduction of myeloid dendritic cells in the blood of donors with NOD2 SNP12 and 13, the maturation of dendritic cells under different culture conditions was analysed.

Although DCs have the ability to induce a T cell response, they can also exhibit tolerogenic properties that might be exploited in the prevention and treatment of e.g. GvHD. Tolerogenic DC are characterized by a semi-mature phenotype with low expression of co-stimulatory B7 molecules. They mainly secrete immunosuppressive mediators, such as IL-10, and lack the production of pro-inflammatory cytokines such as IL-12 [109].

5.4.1. Dendritic cell cultures of donors with NOD2 WT SNP8 and SNP12 and 13

Based on the findings that NOD2 SNP donors have alterations in their myeloid cell compartment and show a reduced amount of mDCs in peripheral blood (chapter 5.1) we hypothesized that the *in vitro* generation of dendritic cells would also be affected in cultures from NOD2 SNP-bearing donors. Due to limited numbers of cells with NOD2 SNPs in heparin-drawn blood samples and more physiological culture conditions we used a protocol for DC generation starting from MNCs instead of purified monocytes [110].

Mononuclear cells (consisting of a mixture of monocytes and lymphocytes) were separated from heparin-drawn blood by ficoll. The isolated cells were cultured in 96-well plates ($1x10^6$ in 100 µl) in RPMI medium supplemented with 10% FCS for 24 hours. After this period the cells were stimulated with or without LPS or MDP for 24 hours.

Dendritic cell maturation markers such as CD80, CD83 and CD86 were significantly up-regulated by LPS in cultures from donors with WT NOD2 but not in cultures from donors harbouring a NOD2 SNP13 mutation indicating that dendritic cell maturation is impaired in these cells. MDP had only a slightly positive effect on dendritic cell maturation. Regarding HLA-DR expression, no significant differences were found independent of the NOD2 status or stimulus (Figure 5.16).

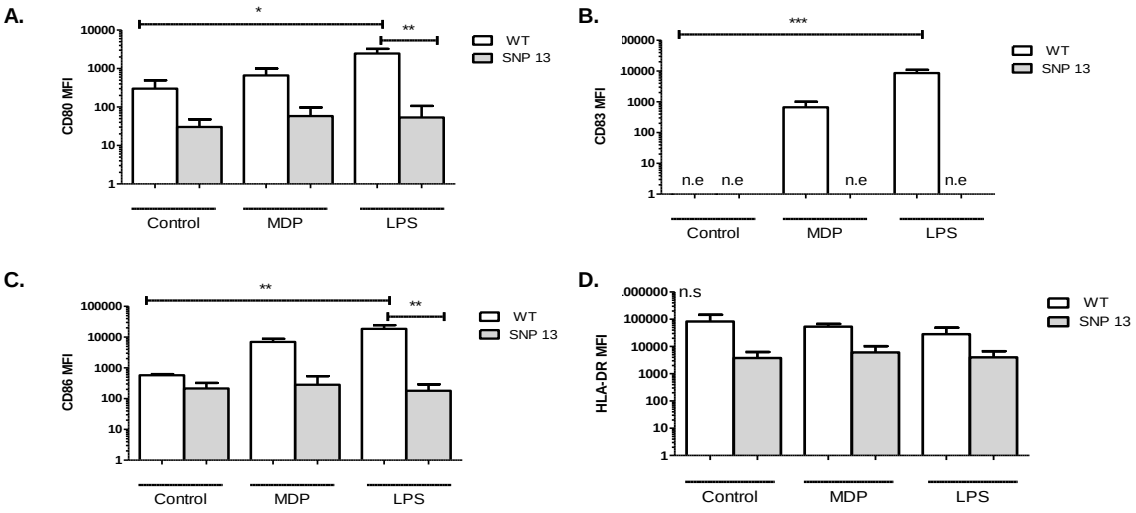

Figure 5.16. SNP13 donors displayed an impaired DC maturation. MNCs were cultured in 96 well plates in RPMI supplemented with 10% FCS, IL-4 and GM-CSF for 24 hours. After this period, cells were stimulated with or without LPS or MDP for additional 48 hours. Shown is the mean of the median fluorescence intensity (MFI) (isotype subtracted) + SEM (*n*=3). Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, n.s.: not significant n.e: not expressed).

Culture supernatants were harvested and analysed by means of ELISA. IL-12 production was not detected in any of the cultures (data not shown). IL-10 analysis revealed that cells were able to produce this cytokine but no difference was found between cell cultures from donors with NOD2 WT and NOD2 SNP 13 (Figure 5.17).

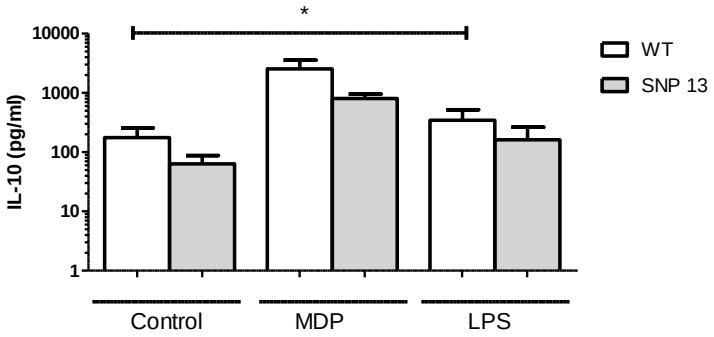

Figure 5.17. IL-10 production is similar in cultures from WT and NOD2 SNP donors. DCs were cultured as previously described. The supernatants were harvested and IL-10 production analysed by means of ELISA. Shown is the mean of 3 experiments + SEM. Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$).

To confirm the data obtained from these MNC-DC cultures, monocytes of WT and NOD2 SNP-bearing donors were isolated via elutriation to generate DCs using the “classical” protocol for DC generation. Therefore monocytes were cultured for 5 days in RPMI medium supplemented with 10% FCS, IL-4 and GM-CSF. Afterwards, cells were stimulated for 48 hours with LPS or MDP. Surprisingly, and contrary to our findings on fast MNC-DC, we found that classical DCs generated from monocytes with NOD2 SNPs expressed higher amounts of CD86 and CD83 upon LPS stimulation compared with WT, indicating an enhanced maturation level of these cells (Figure 5.18). The number of viable cells was not significantly differrent in WT or SNP donors (Figure 5.18 B).

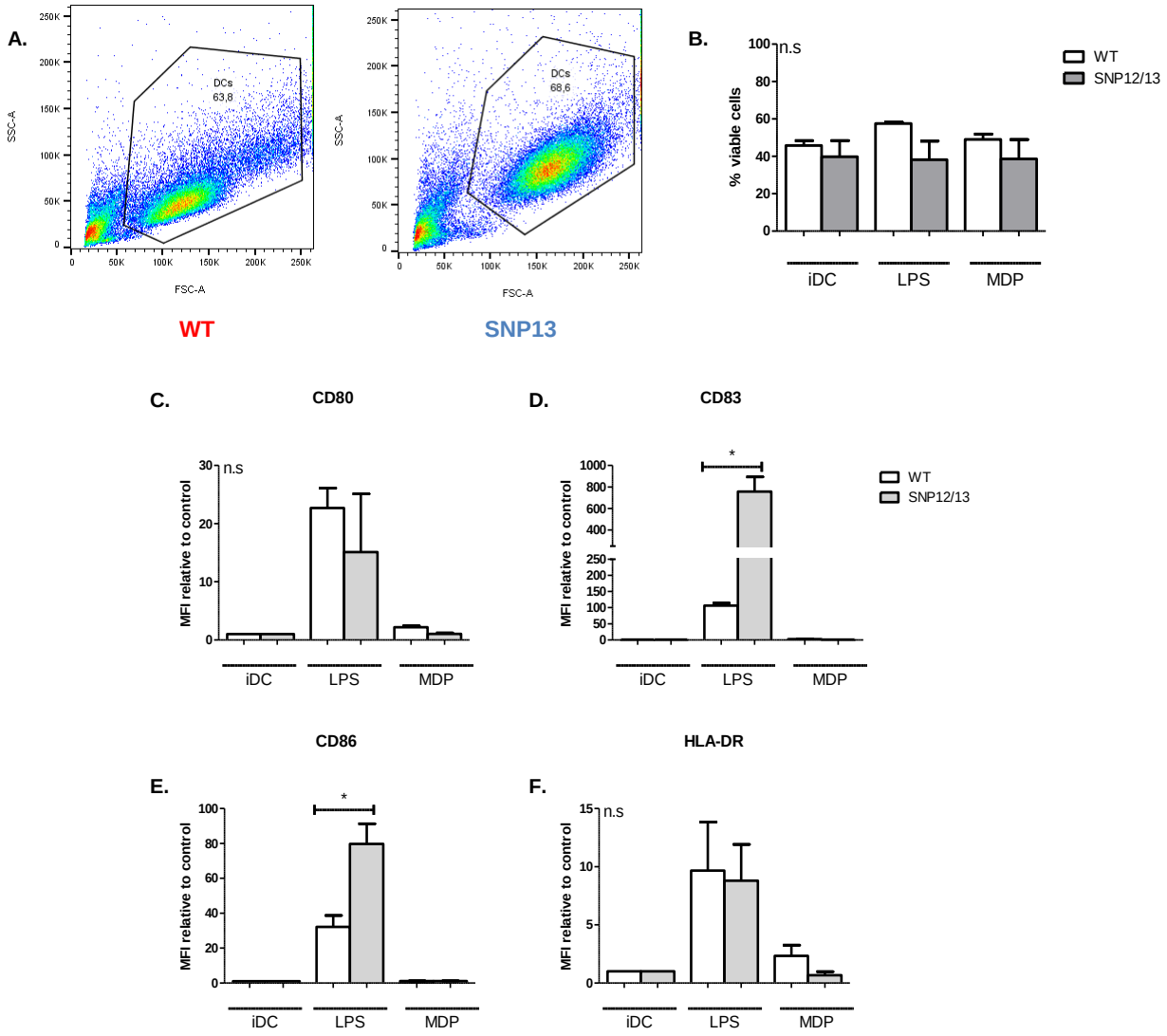

Figure 5.18. SNP12 and 13 donors showed enhanced activation in classical DCs. Monocytes were cultured in flasks in RPMI supplemented with 10% FCS, IL-4 and GM-CSF for 5 days. After this period, cells were stimulated with or without LPS or MDP for additional 48 hours. Shown is one representatice FSC/SSC **(A)** Cell viability **(B)** the mean of the median fluorescence intensity (MFI) **(C-F)** (isotype subtracted) relative to unstimulated cells + SEM (*n*=3). Statistical analysis was performed with Kruskal-Wallis test (*** $p \leq 0.001$, n.s.: not significant).Cell viability was determined with a cell analyser (CASY system).

Culture supernatants were harvested and analysed by means of ELISA. Although not significant, we observed a strong reduction in cytokine production in monocyte- derived DCs generated from SNP12 or 13 donors compared with WT (Figure 5.19).

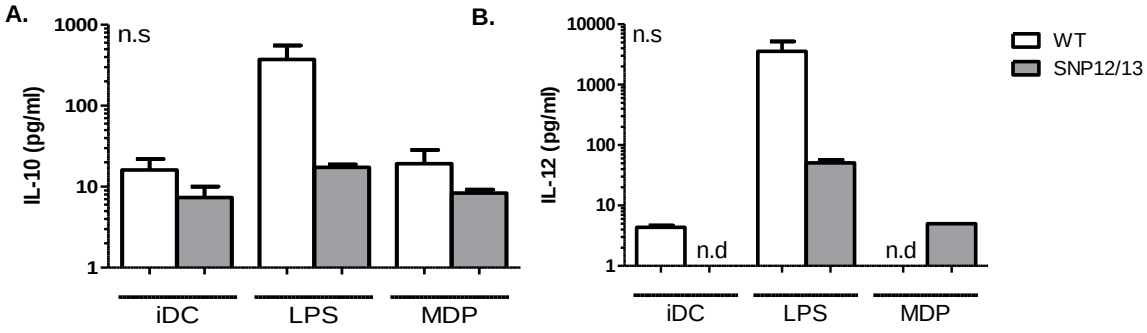

Figure 5.19. IL-10 and IL-12 production is impaired in cultures from NOD2 SNP donors. DCs were cultured as previously described following the classical protocol. The supernatants were harvested and IL-10 and IL-12 production analysed by means of ELISA. Shown is the mean of 3 experiments + SEM. Statistical analysis was performed with Kruskal-Wallis test . n.s: not-significant; n.d- not detected.

5.4.2. Effect of the NOD2-ligand MDP on monocyte-derived dendritic cells

MDP had only little effect on dendritic cell maturation in the previous experiments (Fig. 5.16, Fig. 5.18). We wondered whether this limited capacity to induce maturation is due to the used differentiation protocol or whether it was donor-related. Therefore, the different protocols were applied to the same donor. Here, it was analysed whether MDP can influence dendritic cell maturation in WT donors using different dendritic cell differentiation protocols. Classical DC (cDC) protocols using monocytes (Mo-DCs) and fast DC (fDC) protocols using mononuclear cells (MNC-DC) or monocytes were compared for the generation of dendritic cells. The classical protocol was performed has described above (5.4.1) Alternatively, for the fast protocol, monocytes or mononuclear cells were cultured for 24 hours and then stimulated for another 48 hours. Cells were stained and analysed by means of flow cytometry. Supernatants were harvested, frozen and cytokine production was analysed by ELISA. In contrast to LPS, MDP was incapable to induce the maturation of human monocyte-derived dendritic cells (Figure 5.19 A-D), as MDP failed to up-regulate DC maturation-related markers such as CD80, CD83, CD86 and HLA-DR, independent of the applied protocol. Regarding the LPS treatment, differences in the expression of the maturation markers were observed between the different protocols, however, the differences were not statistically significant. As MDP failed to induce differentiation of dendritic cells, we hypothesised that MDP could induce a semi-mature more tolerogenic phenotype in DCs.

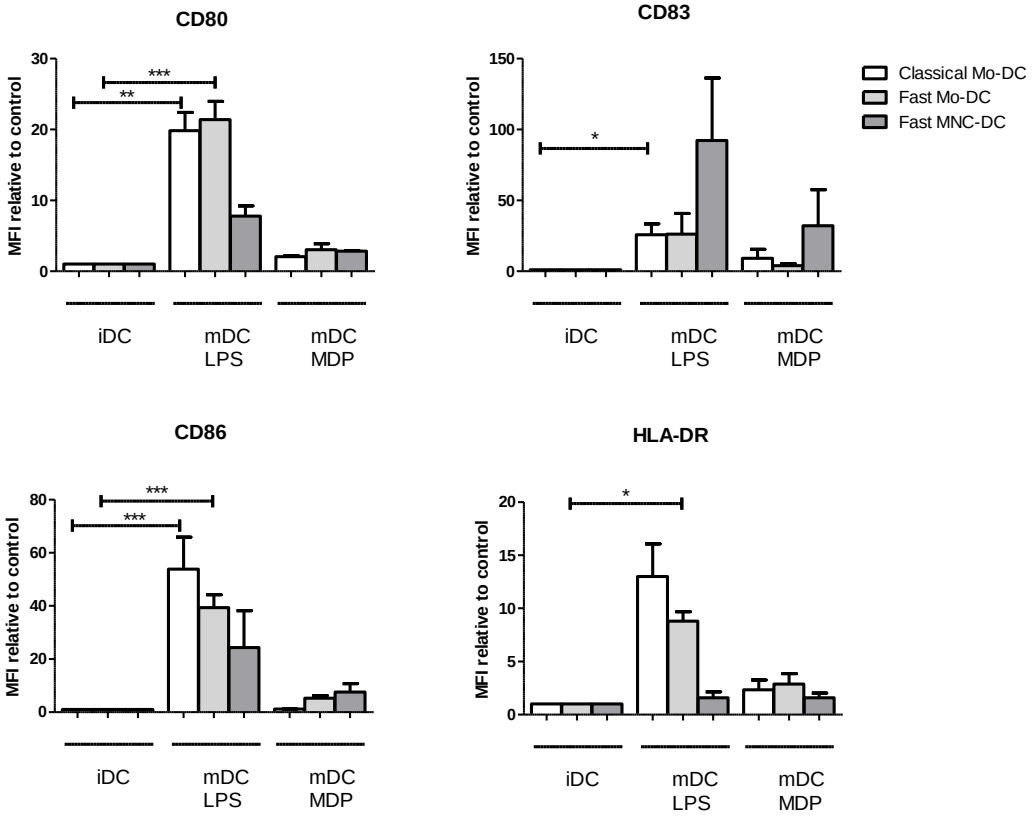

Figure 5.20. MDP is incapable to induce the differentiation of human monocyte-derived dendritic cells (Mo-DCs). Mo-DCs or MNC-DCs were generated under 3 different protocols differing in the initial culture period or initial cell population. For Fast Mo-DC, $10x10^6$ monocytes were cultured for 24 hours. Classical DCs were culture at the same density for 5 days. MNC-DC were generated using $40x10^6$ cultured for 24 hours. Afterwards, all cultures were stimulated for 48 hours and analysed by flow cytometry. Shown is the mean of the median fluorescence intensity (MFI) (isotype subtracted) relative to unstimulated DC + SEM ($n \geq 3$). Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s.: not significant).

5.4.2.1. Impact of MDP on tolerogenic marker expression in DC cultures

It is well-known that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) can induce tolerogenic dendritic cells [111]. Therefore, $1,25(OH)_2D_3$ was used as a positive control for the generation of tolerogenic DC. Monocytes were cultured using the fast protocol and stimulated with or without LPS, MDP and $1,25(OH)_2D_3$. Typical markers for tolerogenic cells were analysed such as ILT2, ILT3 and also PD-L1 and PD-L2, which are co-inhibitory molecules crucial for the down-regulation of T-cell response and maintenance of immune homeostasis.

Data analysis revealed a significant up-regulation of PD-L1 and PD-L2 in DCs stimulated with LPS (Figure 5.21 A and B).

No differences in the expression of immunoglobulin like transcript 2 (ILT2) were found under all the tested conditions (Figure 5.21 A-D). However, ILT3 was significantly increased on DCs stimulated with $1,25(OH)_2D_3$. (Figure 5.21 D). MDP stimulation had a similar effect, although not significant (Figure 5.21 D).

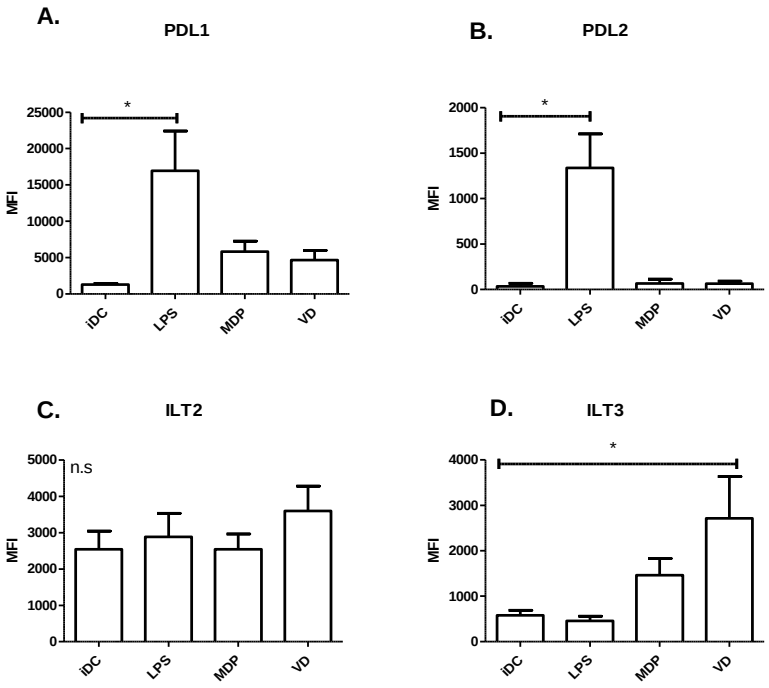

Figure 5.21. Expression of tolerogenic DC markers after culture with LPS, MDP and $1,25(OH_2D_3$. fDC were cultured as previously described and stimulated with or without LPS (100 ng/mL), MDP (100 ng/mL),) and 1,25 $(OH)_2D_3$ ($5x10^{-9}$ M). After 48 h cells were harvested, washed, and stained with the indicated fluorochrome-conjugated monoclonal antibodies for PDL1 (A), PDL2 (B), ILT2 (C) and ILT3 (D) .Samples were analysed by flow cytometry. Shown is the mean of the median fluorescence intensity (MFI) (isotype subtracted). Data are means + SEM (n=3). Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, n.s.: not significant).

5.4.2.2. Cytokine profile of Mo-derived DC *versus* MNC-derived DC

The balance between IL-10 and IL-12 secretion can also determine the T cell response as tolerogenic dendritic cells secrete more IL-10, polarizing the T cell towards a more regulatory phenotype.

To further characterize the generated dendritic cell populations, the supernatants of the cultures were analysed by means of ELISA (Figure 5.22). Comparable to the surface marker analyses, only LPS but not MDP was capable to stimulate cytokine secretion in dendritic cells. This effect was partially dependent on the used differentiation protocol.

Dendritic cells generated from mononuclear cells produced more IL-10 and less IL-12 what indicate that these cells possibly exhibit a more tolerogenic phenotype.

Taking together these results suggest that the presence of lymphocytes in the culture and the duration of the protocol can influence the dendritic cell cytokine secretion profile.

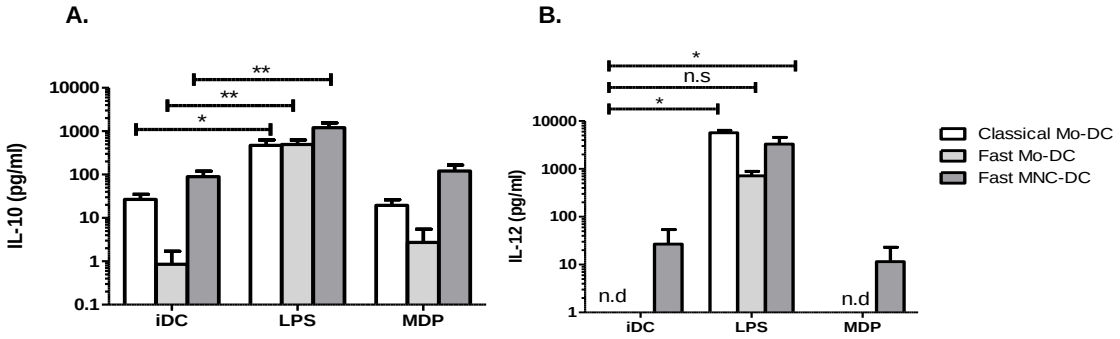

Figure 5.22. IL10 and IL-12 secretion dendritic cells generated under different protocols. After 48 hours supernatants were harvest and the amount of IL-10(A) and IL-12 (B) were determined by ELISA. Data are means + SEM (n ≥4). Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ n.s.: not significant; n.d- not detected).

5.4.2.3. Oxygen consumption and lactate production of Mo-derived DC cultures

The metabolic phenotype has also been implicated in the activation and differentiation of dendritic cells. Stimulation by LPS is known to induce glycolysis and this "glycolytic switch" seems to be important for cell activation and differentiation [112]. Therefore, we investigated whether MDP could alter the metabolic characteristics of dendritic cells. Cells were seeded in 24-well SDR SensorDish Reader plates for 24 hours. Afterwards, cells were stimulated with or without LPS or MDP and placed in the SensorDish Reader for 48 hours to measure oxygen consumption. As expected, LPS reduced respiration in donor 1 and 2 but not in donor 3 (Figure 5.23 A-C). MDP also lowered respiration in donor 1 and 2 but to a lower extent. In addition, lactate was determined in the supernatant of the different cell cultures as readout for glycolysis. In line with the reduced respiration LPS and MDP slightly stimulated glycolysis, however, differences were not significant (Figure 5.23D).

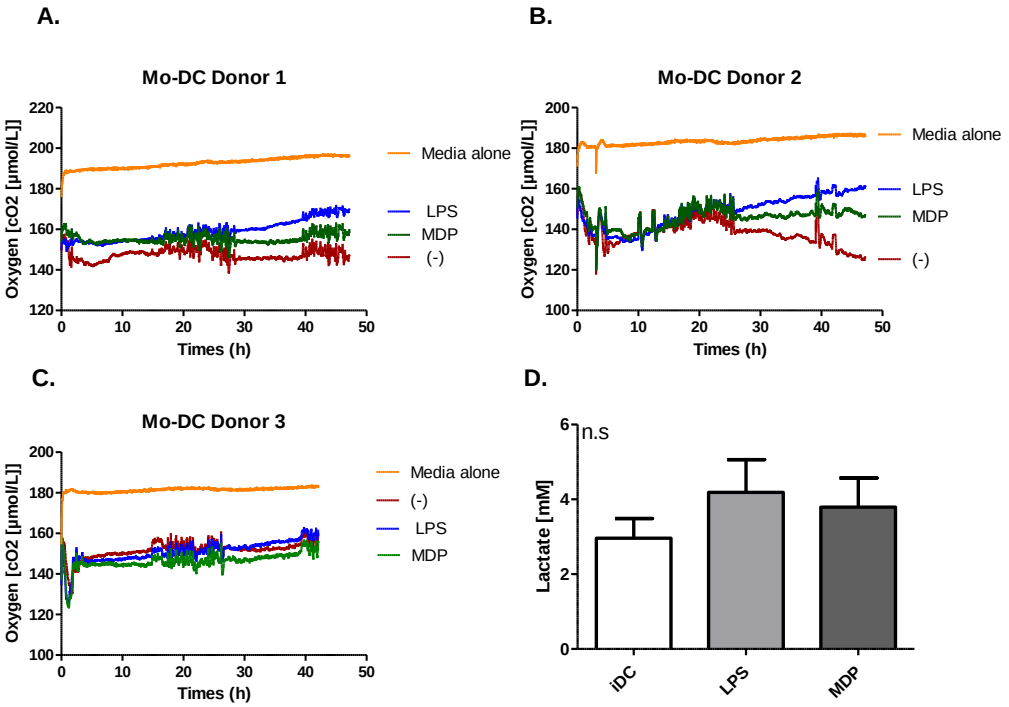

Figure 5.23. Oxygen consumption and lactate production of the Mo-derived DCs were seeded in 24 well SDR SensorDish plates for 24 hours. Afterwards, cells were stimulated with or without LPS or MDP and placed on the SensorDish Reader for 48 hours. For the lactate analysis, supernatants were harvested and lactate determined as described in Chapter 4. Data are means + SEM. N=3. Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) (n.s.: not significant).

5.4.2.4. T cell stimulatory capacity of fast DC *versus* classical DC

To determine the T cell stimulatory capacity of the DCs generated under different protocols, we performed mixed lymphocyte reactions (MLRs) where 10,000 DCs (classical or fast) (previously matured with or without LPS and MDP) were cultured for 5 days with 100,000 allogeneic T cells and the proliferation of the cultures was analysed. In line with the high expression of maturation markers and high capacity for IL-12 secretion, both classical and fast LPS-DC strongly induced proliferation of T lymphocytes, but MDP-DC were incapable to induce T cell proliferation (Figure 5.24).

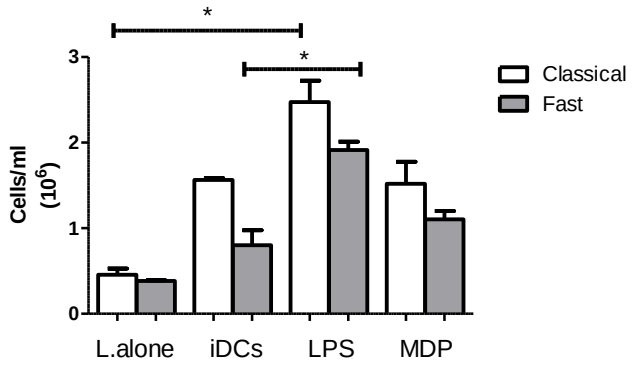

Figure 5.24. Functional analysis of the generated DCs

Monocyte derived-DC generated under different protocols were stimulated for 48 hours with or without LPS (100 ng/mL) or MDP (100 ng/mL) under different protocols. DCs were harvested and washed.10,000 DCs (cDCs or fDCs) were co-cultured with allogeneic T lymphocytes.L. alone refers to the unstimulated lymphocyte culture. After five days, the proliferation rate of T cells was analysed. Shown are means + SEM (n = 3). Statistical analysis was performed using Kruskal-Wallis test. (* $p \leq 0.05$).

5.4.2.5. Generation of regulatory T cells in the MLR

Regulatory T cells are a population of T cells with immunosuppressive activity and can be identified by high levels of CD25 and the expression of the transcription factor FOXP3. This cell population is of great importance in maintaining peripheral tolerance [66]. Regulatory T cells can be induced e.g. via culturing naïve T cells with tolerogenic DCs. To analyse the lymphocyte populations after culturing dendritic cells with allogeneic T cells, cells were harvested, stained for CD4, CD25 and FOXP3 and analysed by means of flow cytometry. Co-culturing T cells with iDCs differentiated applying the fast protocol or the classical protocol did not result in any difference in $FOXP3^+$ T cells (Figure 5.25). Immature DCs and MDP-stimulated DCs resulted in similar numbers of $FOXP3^+$ cells whereas cultures with LPS-stimulated DCs had reduced numbers of $FOXP3^+$ cells

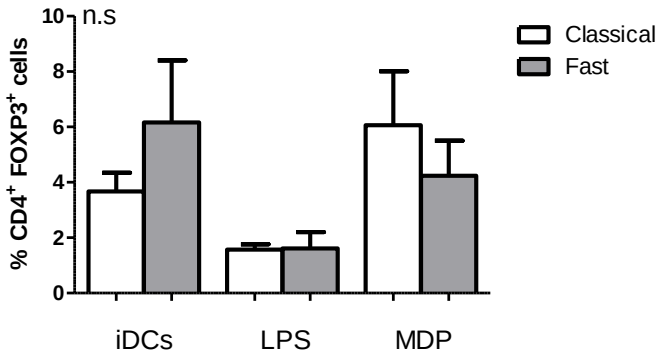

Figure 5.25. Amount of FOXP3$^+$ CD4$^+$ cells after co-culturing with DCs matured either with MDP ord LPS Monocyte derived-DC, differentiated applying the classical or fast protocol, were stimulated for 48 h with or without LPS (100 ng/mL) or MDP (100 ng/mL). Cells were harvested and washed.10,000 DCs (cDCs or fDCs) were co-cultured with allogeneic T lymphocytes in the absence of LPS or MDP. After five days, the proliferation rate of T cells was analysed and the cells stained for CD4 and Foxp3. Data are means + SEM (n = 3). Statistical analysis was performed using Kruskal-Wallis test. (n.s. not significant).

5.5. Impact of vitamin D_3 on immune cells

5.5.1. Effect of vitamin D_3 on human monocytes

5.5.1.1. Vitamin D3 and NOD2 interplay

Beside NOD2 polymorphisms, vitamin D_3 receptor gene polymorphisms have also been associated with GvHD [96]. Furthermore, it was already demonstrated that vitamin D_3 deficiency is a common phenomenon in allogeneic transplant patients and may also be involved in the pathogenesis of GvHD [89]. Although at the first glance NOD2 and vitamin D_3 appear to be completely independent factors, they seem to be linked since the active metabolite of vitamin D_3, 1,25$(OH)_2D_3$, stimulates the expression of the pattern recognition receptor NOD2/CARD15 on monocytes [113].

In order to investigate whether physiological concentrations of 25$(OH)D_3$ and 1,25$(OH)_2D_3$ are also capable to induce NOD2 mRNA expression, human monocytes were incubated for 24 hours with or without vitamin D_3 and stimulated for additional 24 hours with or without LPS, MDP and combination. Next, total cellular mRNA was isolated and gene expression was analysed by means of real-time quantitative PCR (RT-qPCR).

Although not significant, a trend towards an up-regulation of *NOD2* mRNA expression by 25-hydroxyvitamin D3 (25$(OH)D_3$) and 1,25$(OH)_2D_3$ (Figure 5.23) was observed under all treatments, with the exception of the combined treatment with LPS and MDP.

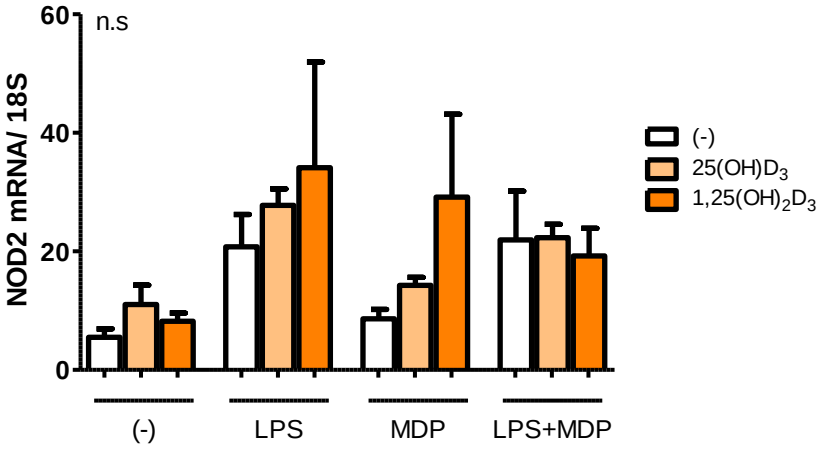

Figure 5.26. NOD2 mRNA expression in human monocytes. Human monocytes were incubated with or without 25(OH)D_3 ($5x10^{-8}$ M) or 1,25$(OH)_2D_3$, ($5x10^{-9}$ M) for 24 hours. Afterwards, cells were further stimulated with LPS (100ng/ml), MDP (100 ng/ml) or the combination for another 24 hours. Subsequently, cells were lysed, RNA was isolated and NOD2 mRNA analysed by means of qRT-PCR. Data are means ± SEM (n =3). Statistical analysis was performed with Kruskal-Wallis test (n.s.:not-significant).

5.5.2. Effect of Vitamin D_3 on human T cells

5.5.2.1. FoxP3 induction by 25(OH)D_3 in human $CD4^+$ T cells

The tolerogenic effects of vitamin D_3 on myeloid cells have already been extensively studied [111, 114, 115]. It is widely accepted that 1,25$(OH)_2D_3$ has an indirect DC-mediated effect on T cells [116]. 1,25$(OH)_2D_3$-treated DCs are capable to induce FOXP3 expression in T cells, a marker for regulatory T cells. However, little is known about the direct impact of 25(OH)D_3 and 1,25$(OH)_2D_3$ on FOXP3 expression in T cells. Furthermore, the majority of these studies use high, non-physiological concentrations of vitamin D_3. Here, the impact of 25(OH)D_3, the most important serum metabolite due to its stability, was investigated. $CD4^+$T cells were isolated from human PBMCs using magnetic bead separation and stimulated with anti-CD3/CD28 T cell expander beads (TCE) in different ratios (1 bead/T cell or 1 bead /10 T cells) in the presence or absence of 25(OH)D_3 for 3 days. After this period, cells were harvested, counted and stained for FOXP3. Data analysis showed an increase in FOXP3 expression in the presence of 25(OH)D_3 independent of the number of stimulator beads, demonstrating that physiological concentrations of the vitamin D_3 precursor are able to induce a regulatory phenotype in T cells independent of the presence of DCs (Fig. 26). A lower level of stimulation with 0.1 bead/T cell led to a significantly decreased T cell proliferation independent of 25(OH)D_3.

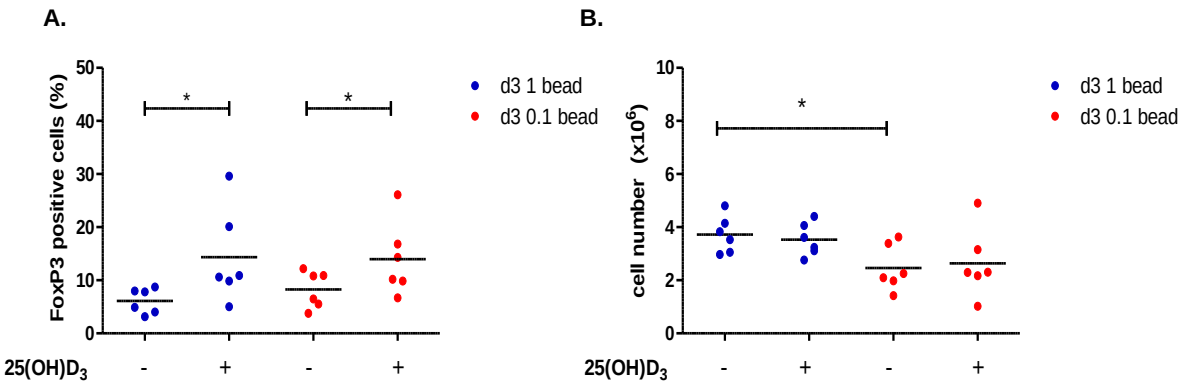

Figure 5.27. Amount of FOXP3+ cells (A) and cell proliferation (B) in $CD4^+$ T cell cultures. 0,5x10^6 cells were cultured in 24 well plates in the presence or absence of 25(OH)D_3 and stimulated with different TCE bead: cell ratios (1 bead:1 cell or 1 bead: 10 cells) . The amount of $FOXP3^+$ cells was analysed by flow cytometry. Each dot represents one individual donor. Shown is the mean (n=6). Statistical analysis was performed with repeated measures ANOVA (* $p \leq 0.05$).

5.5.2.2. FoxP3 induction by Vitamin D_3 in naïve $CD4^+$ T cells

In the previous experiments, an increase in FOXP3 expression was found in the presence of 25(OH)D_3. However, the initial culture contained not only naïve $CD25^-$T cells but also $CD25^+$ T cells, meaning that the observed increase in $FOXP3^+$ cells could be due to the expansion of a pre-existing population of regulatory T cells. To overcome this, cells were FACS sorted for $CD4^+CD25^-$ T cells (kindly performed by AG Hoffmann/Edinger, Department of Internal Medicine III, University Hospital Regensburg) to assure that the observed results represent an induction of CD25 and FOXP3.

In this set of experiments, the impact of both forms of vitamin D_3, the precursor, (25(OH)D_3) and the active form (1,25(OH)$_2D_3$), were studied in naïve $CD4^+$ T cells. TGF-β, a well-known stimulus for FOXP3 induction was used as a positive control [117].

Analysis revealed that all stimuli added to the cultures were able to significantly up-regulate FOXP3 expression (Figure 5.27), independently of the presence of DC. A significant up-regulation of FOXP3 was also found in the presence of 25(OH)D_3 indicating either a direct induction of $FOXP3^+$ T cells by the vitamin D_3 precursor or the hydroxylation of 25(OH)D_3 to 1,25(OH)$_2D_3$ by T cells.

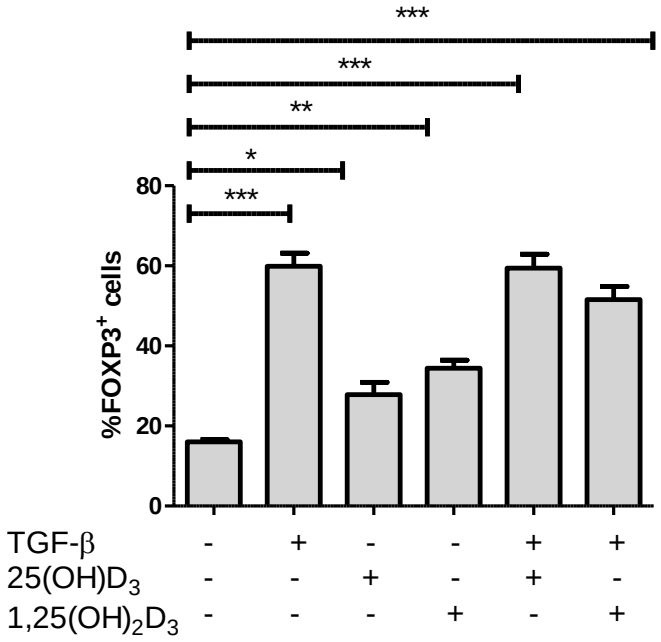

Figure 5.28. FOXP3 induction by $1,25(OH)_2D_3$ and $25(OH)D_3$ in comparison to TGF-β,. Naïve CD4+ T cells were stimulated with TGF-β (10 ng/ml), $25(OH)D_3$ ($5x10^{-8}$ M) and $1,25(OH)_2D_3$, ($5x10^{-9}$ M) in 24 well plates for 4 days in the presence of TCE beads (1 bead/cell). The amount of $FOXP3^+$ cells was analysed by flow cytometry. Data are means ± SEM (n ≥ 5). Statistical analysis was performed with one-way ANOVA (* p ≤ 0.05, *** p ≤ 0.001).

FOXP3 expression was also analysed on the mRNA level by means of qRT-PCR (Figure 5.29). Surprisingly, a significant increase of FOXP3 m RNA was only found for the TGF-β and $1,25(OH)_2D_3$ combination.

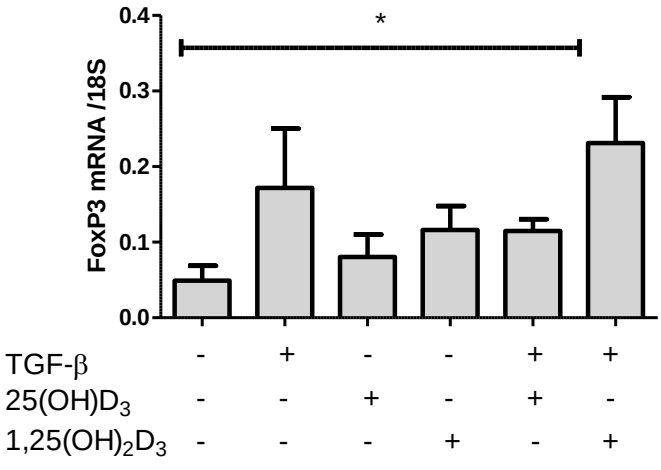

Figure 5.29. FOXP3 mRNA expression is increased upon TGF-β + 1,25 $(OH)_2D_3$ treatment. Cells were cultured with the indicated stimuli for 4 days. Afterwards, cells were lysed, RNA was isolated and FoxP3 mRNA analysed by means of qRT-PCR. Data are means + SEM (n ≥2). Statistical analysis was performed with Kruskal-Wallis test (* p ≤ 0.05).

There is some evidence that T cells also express CYP27B1, the enzyme catalysing the hydroxylation of $25(OH)D_3$ to $1,25(OH)_2D_3$. However, data on the production of $1,25(OH)_2D_3$ from $25(OH)D_3$ by T cells are inconsistent [118-120].

Since the expression of *CYP27B1* in T cell cultures suggest that the cells are perhaps able to produce $1,25(OH)_2D_3$, culture supernatants were harvested after 4 days and analysed for the level of $1,25(OH)_2D_3$. An increase in $1,25(OH)_2D_3$ was found in the presence of 50 nM $25(OH)D_3$ and significantly higher levels of $1,25(OH)_2D_3$ were detected upon incubation of T cells with 100 nM $25(OH)D_3$. (Figure 5.30).

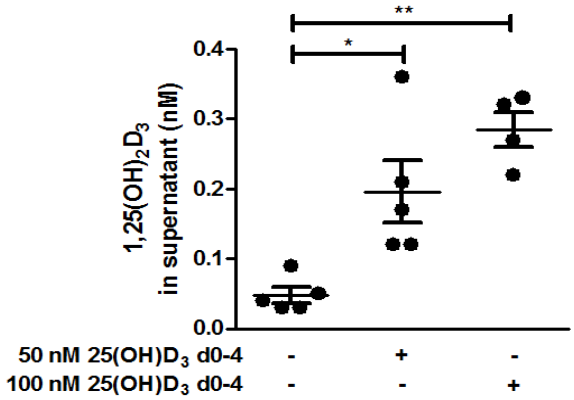

Figure 5.30. Amount of $1,25(OH)_2D_3$ in the culture supernatant of human CD4+ T cells. Cells were cultured for 4 days in the presence of $25(OH)D_3$ and TCE beads. After the culture period, supernatants were harvested and the amount of $1,25(OH)_2D_3$ determined by using a radioimmunoassay according to the manufacturer's instructions (Immunodiagnostic Systems). Data are means + SEM ($n \geq 4$). Statistical analysis was performed with one-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$,).

5.5.2.2.1. Cytokine secretion by T cells in the presence of vitamin D_3

The effect of $1,25(OH)_2D_3$ on T cell responses is not limited to the induction of regulatory T cells. It is discussed that $1,25(OH)_2D_3$ inhibits the Th1 and Th17 response[79].Therefore, the secretion of soluble CTLA-4 and other cytokines in T cell cultures were analyzed in the presence or absence of $25(OH)D_3$ or $1,25(OH)_2D_3$ after 4 days stimulation with TCE. A significant increase in the secretion of soluble CTLA-4 was detected in the presence of TGF-β in combination with $1,25(OH)_2D_3$ (Figure 5.31 A), as well an increase in IL-10 production (Figure 5.31 B). Although not significant, the stimulation of $CD4^+CD25^-$ T cells in the presence of $25(OH)D_3$ lead to the down-regulation of IL-17 (Figure 5.31 C) and IFN-γ secretion (Figure. 5.31 D), indicating that also other T cell populations than $FOXP3^+$ T cells might be influenced by $25(OH)D_3$.

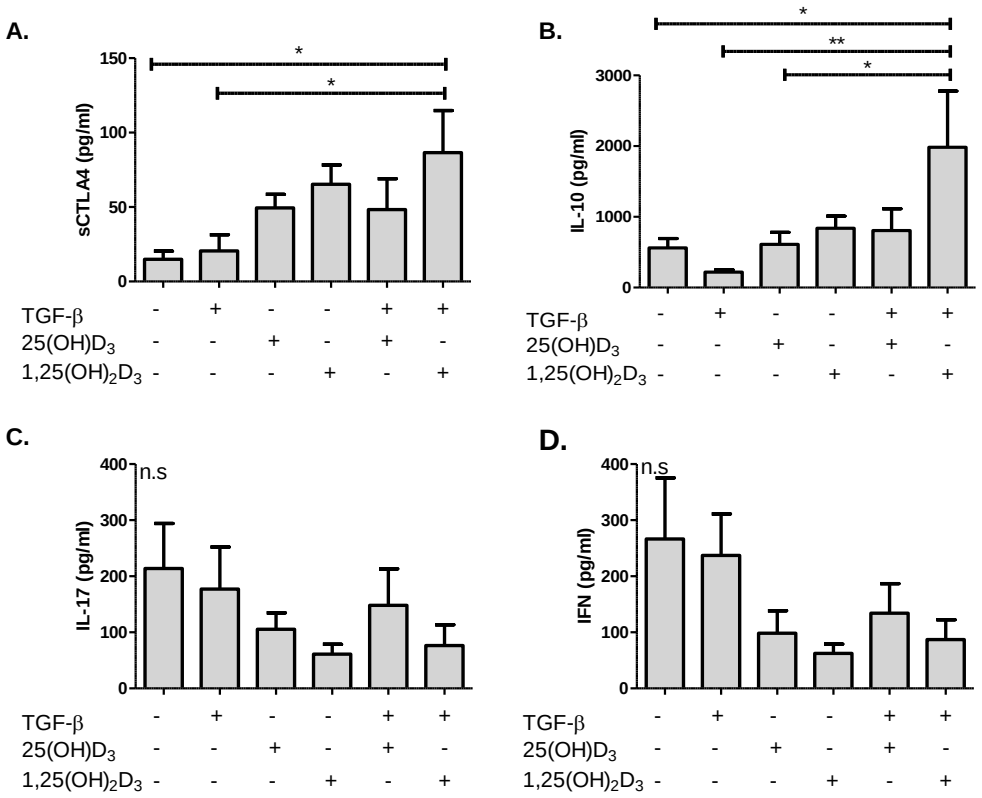

Figure 5.31. Cytokine profile of $CD4^{+}25^{-}$ T cells stimulated with TGF-β, $25(OH)D_3$ or $1,25(OH)_2D_3$. Cells were cultured with the indicated stimuli for 4 days. Afterwards, supernatants were harvested and cytokines measured by means of ELISA. Amount of soluble CTLA-4 **(A)**,IL-10 **(B)**, IL-17 **(C)** and IFN (D) in culture supernatant. Data are means + SEM (n ≥3). Statistical analysis was performed with one-way ANOVA (A and B) or Kruskal-Wallis test (C and D). (* $p \leq 0.05$, ** $p \leq 0.01$).

5.6. In vivo effects of vitamin D_3

Based on our own *in vitro* data and the available information regarding vitamin D_3 effects on the immune system, we decided to establish a model of vitamin D_3 sufficiency *versus* deficiency in order to investigate vitamin D_3 modulation of the immune system *in vivo.*

5.6.1. Vitamin D_3 sufficient *versus* deficient mouse model

Vitamin D_3 levels are strictly regulated and relatively stable. Therefore, it is necessary to use young mice directly after weaning to generate mice with low $25(OH)D_3$ levels. Two independent experiments were conducted, using 3 weeks old female BALB/c mice purchased from Charles River Laboratories. Mice were fed with a special vitamin D_3-deficient (<100 U/Kg) or vitamin D_3-sufficient (1500 U/Kg) diet (Ssniff GmbH). Serum $25(OH)D_3$ levels were measured by means of liquid chromatography high-resolution tandem mass spectrometry in the Department of Clinical Chemistry (Figure 5.32).

In a third experiment we used an outbred mouse model (CD-1) which should more closely mimic the situation in patients. Outbred mice have unpredictable genotypes and therefore are expected to have a diverse population of phenotypes [121].

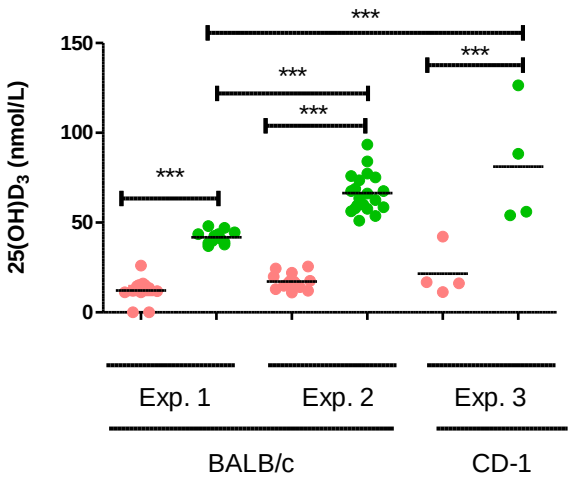

Figure 5.32. Serum vitamin D_3 levels of BALB/c mice (experiments 1 and 2) and CD1 mice (experiment 3) at the age of 10 weeks. Mice were fed with vitamin D_3 deficient diet (pink), or with vitamin D_3 sufficient (green). Blood samples were collected from the animal´s tail vein in three independent experiments. The blood was centrifuged and the serum collected and frozen until analysis. Each symbol represents an individual mouse. Statistical analysis was performed with one-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. n.s not significant).

Regarding BALB/c mice in both experiments (1 and 2), significantly lower values of serum 25(OH)D_3 were obtained in mice receiving the vitamin D_3 deficient diet (mean 41.9 nM (suf.) *vs.* 12.2 nM (def.) for experiment 1 and 66.6 nM *vs.* 17.2 nM in experiment 2).

In the case of outbred CD-1 mice, we also observed a significant difference in the 25(OH)D_3 serum levels in mice receiving different diets. Here, a mean of 81.18 nM 25(OH)D_3 was detected in the serum of vitamin D_3-sufficient diet mice compared with 21.60 nM 25(OH)D_3 for the vitamin D_3-deficient diet.

Due to the different vitamin D_3 levels in animals fed with the vitamin D_3-sufficient diet in experiments 1 and 2, data were analyzed separately.

5.6.1.1. Impact of the serum 25(OH)D_3 level on immune cell composition

5.6.1.1.1. Impact on the main cell populations

To investigate the impact of serum vitamin D_3 levels on the immune cell composition, mice were sacrificed at the age of 17-18 weeks and their blood, spleen and bone marrow were examined by flow cytometry.

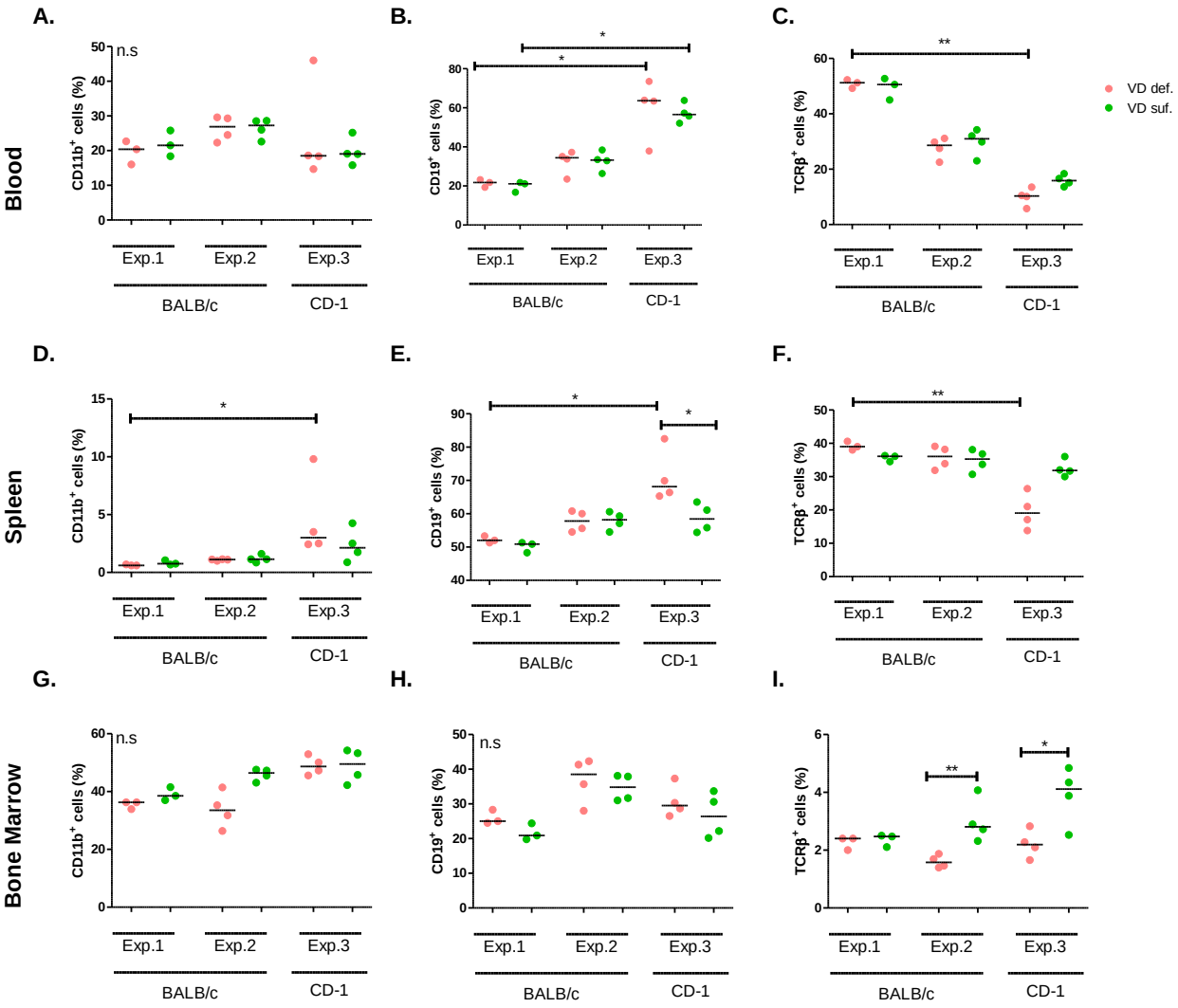

Figure 5.33. Immune cell composition of BALB/c and CD-1 mice. (A-C) Quantification of myeloid cells (A), B-Cells (B) and T Cells (C) among living mononuclear cells in the blood of healthy BALB/c and CD-1 mice fed with different Vitamin D_3 diets. (D-F). Quantification of myeloid cells (D), B-Cells (E) and T Cells (F) in spleen cells as described in (A-C). (G-I) Quantification of myeloid cells (G), B-Cells (H) and T Cells (I) in bone marrow cells as described in (A-C). Each symbol represents an individual mouse and horizontal lines indicate the median. Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. n.s not significant).

Regarding the myeloid compartment, mice with high or low serum 25(OH)D_3 level exhibited no significant differences in the amount of $CD11b^+$ cells in the blood and spleen, irrespective of the used mouse strain. Concerning B cells, (Figure 5.33 B,E,H) we also found no significant difference in $CD19^+$ cells between mice with high or low serum 25(OH)D_3 levels. Only CD-1 mice with low 25(OH)D_3 levels showed fewer $CD19^+$ B cells in the spleen. Overall more differences were found between the different mouse experiments than between mice fed vitamin D_3 sufficient vs. deficient diets. The only immune cell population with relative differences related to vitamin D_3 levels seems to be the T cell population (Figure 5.33 C,F,I). Here, increased levels of T cells were found in mice with higher 25(OH)D_3 levels in bone marrow in experiment 2 and 3.

5.6.1.1.2. Impact of 25(OH)D_3 serum level on immune cell sub-populations

Although differences in the main immune cell populations were hardly detected between mice receiving a vitamin D_3 sufficient vs. deficient diet, effects on sub-populations cannot be excluded. To better characterize the immune cell composition of the mice, different immune cell sub-populations were analyzed by means of flow cytometry.

Regarding the myeloid compartment, we observed an increase in myeloid-derived suppressor cells (MDSC, $CD11b^+Gr^+$) in the blood and bone marrow (significant only experiment in experiments 2 and 3) but not in the spleen of mice fed with sufficient levels of vitamin D_3 in experiments 1 and 2 (Figures 5.34 A and D).

Concerning $CD8^+$ T cells (Figures 5.34 C, F and I), an increase in this population was detected in CD-1 mice in the spleen and bone marrow of mice fed with sufficient levels of vitamin D_3. No vitamin D_3-related differences in the number of $CD4^+$ T cells were detected in blood, spleen and bone marrow (Figures 5.34 B, E and H).

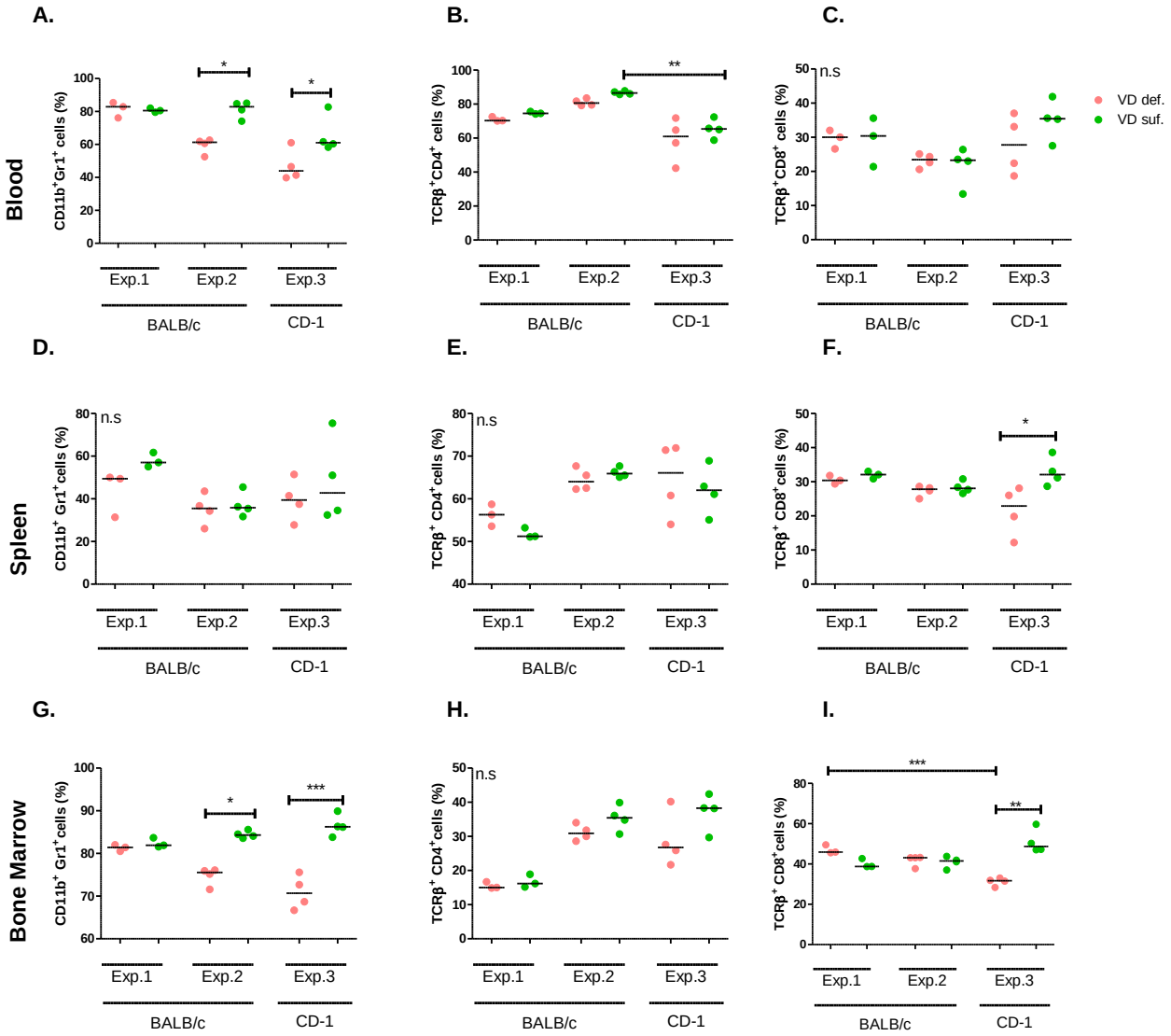

Figure 5.34. Analysis of immune cell subpopulations in the blood, spleen and bone marrow of BALB/c and CD-1 mice. Quantification of MDSC(A, D, G), $CD4^+$ T cells (B, E,H) and $CD8^+$ T Cells (C.F,I) among living mononuclear cells healthy BALB/c and CD-1 mice fed with different vitamin D_3 diets. Each symbol represents an individual mouse and horizontal lines indicate the median. Statistical analysis was performed with Kruskal-Wallis test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. n.s not significant).

Since a positive relation between vitamin D_3 levels and $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs is described in patients and we found a positive effect of vitamin D_3 on the induction of $Foxp3^+$ cells *in vitro*, this cell population was also analyzed in the blood, spleen and bone marrow of mice. In BALB/c mice vitamin D_3 levels did not significantly influence the Treg population whereas in CD-1 mice high vitamin D_3 levels even down-regulated regulatory T cells in the blood. Helios is a transcription factor expressed in a subset of $Foxp3^+$ Tregs. It has been proposed that Helios is a marker of thymic derived Treg (tTreg). Other studies have suggested that Helios is primarily a marker of T cell activation [122]. Only in CD-1 mice $Foxp3^+Helios^+$ cells were significantly suppressed in the blood of mice with sufficient vitamin D_3 levels (Figure 5.34 B). In contrast, $CD4^+Foxp3^-$ $Helios^+$ cells were

enriched in the bone marrow of BALB/c mice with sufficient vitamin D_3 (experiment 2) levels and a trend was also observed in the blood (experiments 2 and 3).

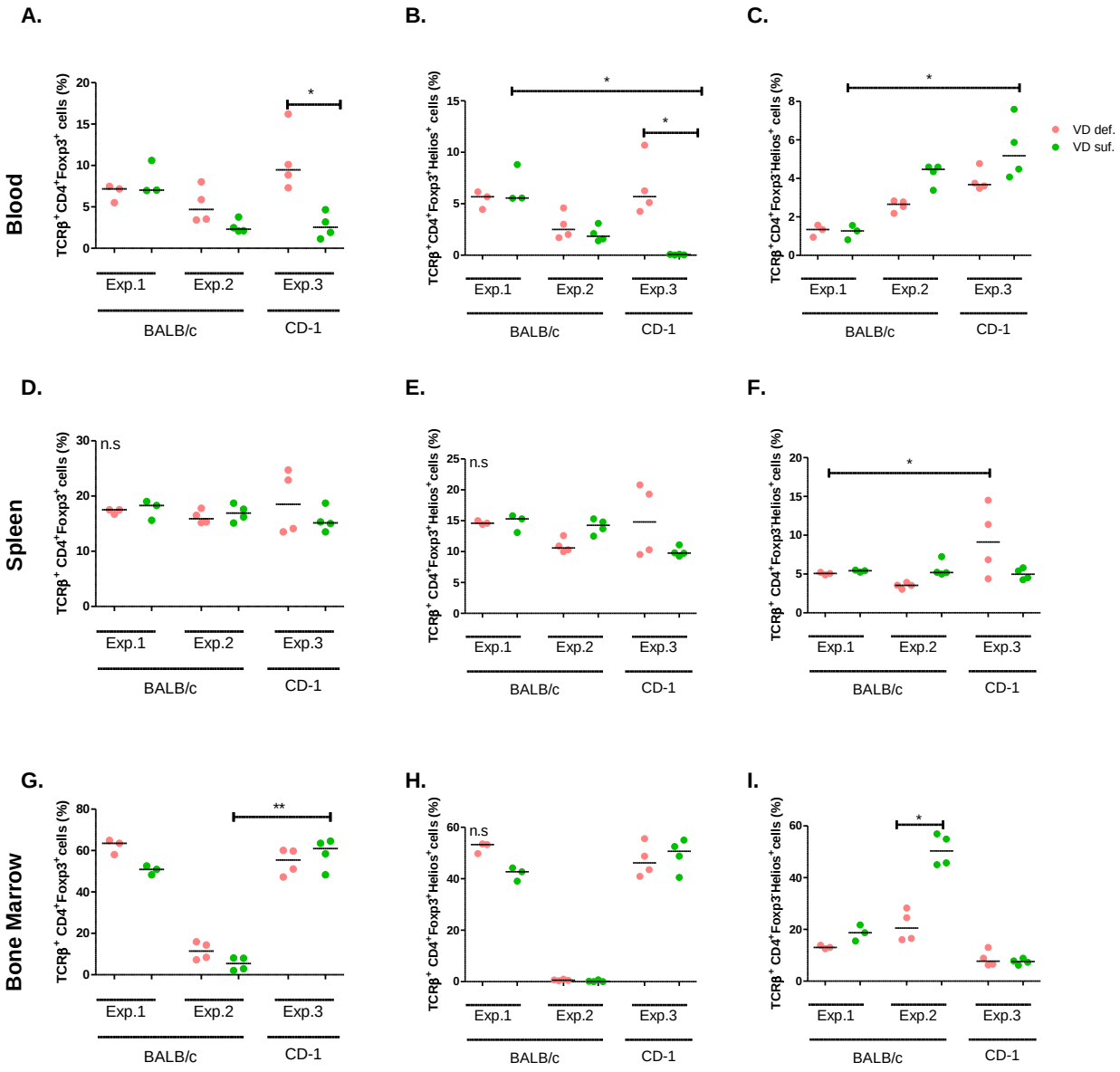

Figure 5.34. Analysis of subpopulations in the blood, spleen and bone marrow of BALB/c and CD-1 mice. Quantification of $CD4^+Foxp3^+$ T cells (A, D, G), $CD4^+$ $Foxp3^+Helios^+$ T cells (B, E,H) and $CD4^+$ $Foxp3^-Helios^+$ T cells (C.F,I) among living mononuclear cells healthy BALB/c and CD-1 mice fed with different vitamin D_3 diets. Each symbol represents an individual mouse and horizontal lines indicate the median. Statistical analysis was performed with Kruskal-Wallis test(* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. n.s not significant).

5.6.1.2. Impact of 25(OH)D_3 serum level on gut microbiota

The role of microbiota in health and disease has been acknowledged in recent years, and several studies suggest an important interplay between the microbiome and the immune response [123-125].

In particular, dysbiosis, an imbalance in microbiota composition, is postulated to be a key factor in human diseases, such as inflammatory bowel disease and GvHD [47, 126, 127].

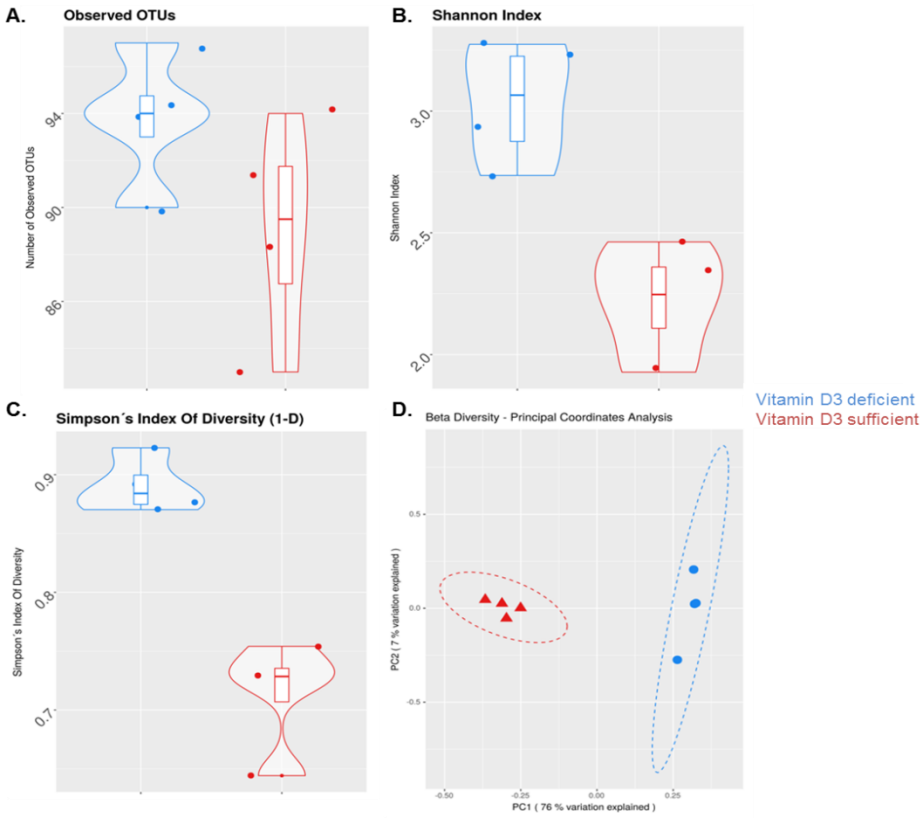

Figure 5.35. Mice fed a vitamin D_3 deficient diet have higher microbiota diversity. The microbiota of mice fed with Vitamin D_3 sufficient and deficient diet were determined by 16rDNA sequencing. The microbial diversity was analysed and shown as Observed Operational Taxonomic Unit (OTU) (A), Shannon Index (B), Simpson´s Index (C) and the difference between the two groups by principal component analysis (D).

As several studies also suggest an impact of vitamin D_3-related SNPs and vitamin D_3 serum status on the microbiota composition [123], we hypothesized that mice fed under different vitamin D_3 diets would exhibit a different gut microbiota composition. In order to analyze gut microbiota composition of the mice, stool samples were collected and analyzed by means of 16S ribosomal DNA (rDNA) PCR analysis (in collaboration with Dr. Hiergeist and Prof. Dr. Dr. André Gessner, Institute for Medical Microbiology and Hygiene, University Hospital Regensburg).

Surprisingly, higher microbial diversity was found in mice receiving the vitamin D_3-deficient diet (Figure 5.35 A-C). The relative abundance of microbiota genera was also found to be significantly different in mice receiving the vitamin D_3-deficient or -sufficient

diets. Mice fed with one diet grouped nicely, as shown in the principal component analysis (Figure 5.35D).

The most striking difference separating the two groups were the high levels of *Akkermansia* microbes in the mice fed with a vitamin D_3-sufficient diet compared to mice fed with a vitamin D_3-deficient diet (Figure 5.36). On the other hand, mice receiving the vitamin D_3-deficient diet showed increased numbers of other microbial genera, namely *Anaerotruncus*, *Bacteroides* and *Oscillibacter*. Taken together, these data demonstrate that vitamin D_3 plays a role in the composition of the gut microbiota, with low levels of vitamin D_3 completely abolishing presence of *Akkermansia* in the murine intestine.

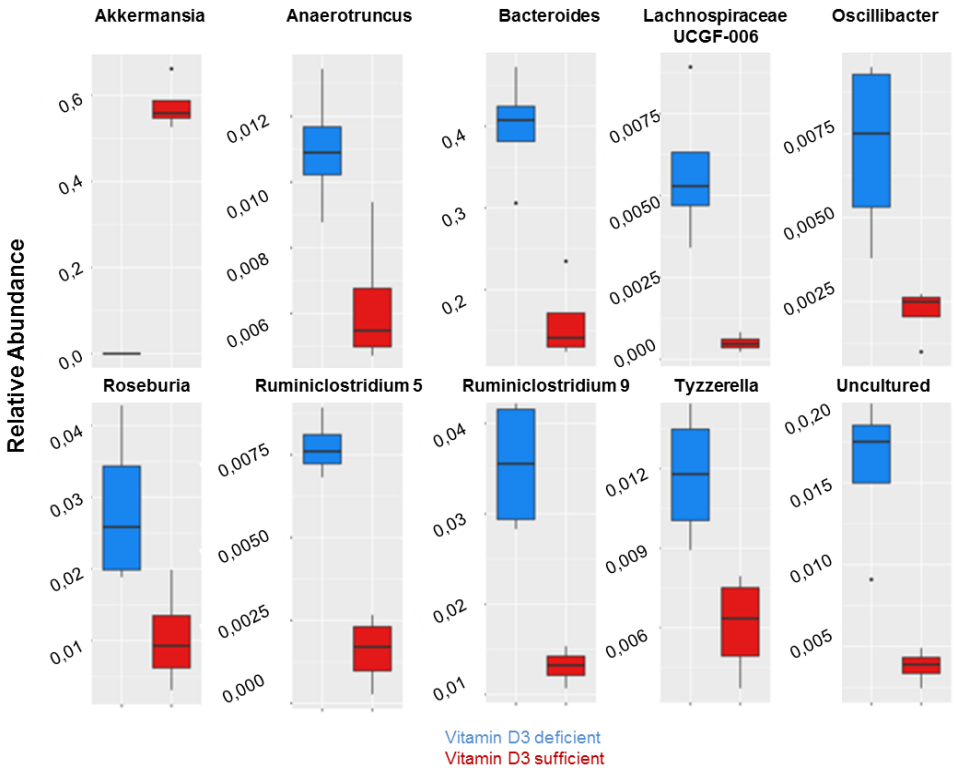

Figure 5.36. The ten most abundant bacterial genera in stool samples. The microbiota of mice fed with vitamin D_3 sufficient or deficient diet was determined by 16rDNA sequencing diet.(n=4/group).

6. Discussion & Perspectives

6.1. Impact of the NOD2 status on immune cell composition in human blood

Nucleotide-binding oligomerization domain containing 2 (NOD2) is an intracellular pattern recognition receptor involved in detection of bacteria [37] and single nucleotide polymorphisms (SNPs) of NOD2 have been linked to several inflammatory disorders, such as Crohn's disease (CD), Blau syndrome , early-onset sarcoidosis and Graft-versus-Host disease (GvHD) [128]. These findings indicate that this receptor is of crucial importance for maintaining immune cell homeostasis, especially in the gastrointestinal tract.

Although it is known that NOD2 is expressed in different cell types such as paneth cells [129], epithelial cells [130] and keratinocytes [131], it is widely accepted that the highest NOD2 expression is found in myeloid cells. Several publications describe alterations in the function and differentiation of myeloid cells with SNPs of NOD2 most in patients with Crohn's disease [132-134]. However, to our knowledge nothing is known about changes in the immune cell composition in healthy persons or patients with NOD2 SNPs. Furthermore, there is also limited knowledge about functional aspects of these mutations in healthy donors. This may be related to the fact that the frequency of NOD2 mutations such as SNP13 is relatively rare (ca 5 % of healthy donors carry the mutation) and it is difficult to obtain sufficient samples for analyses.

Analysing the blood cell composition of healthy donors bearing NOD2 SNPs 8, 12 and 13, we detected only significant changes in the myeloid compartment. Since this blood cell population is the one with highest expression of this intracellular receptor, it seems reasonable that the most striking differences were found in monocytes and dendritic cells (DCs).

Flow cytometry analysis of mononuclear cells separated from blood samples collected with heparin tubes revealed that donors bearing NOD2 SNP12 and SNP13 had less $CD33^+$ cells, a common myeloid marker, indicating that NOD2 could be involved in the regulation of haematopoiesis. In further studies it would be interesting to analyse neutrophil counts in blood, as neutrophils represent the main myeloid blood cell population.

Additionally, we detected an increased CD16 expression on monocytes in the blood of NOD2 SNP12- and SNP13-bearing donors, compared to WT and SNP8 donors. Also, myeloid DCs were reduced in SNP donors compared with WT, further supporting the evidence for changes in the myeloid compartment of these donors.

Recently, it has been demonstrated that NOD1 is involved in the steady-state survival and turnover of circulating neutrophils and inflammatory monocytes in mice [135]. Therefore, it is seems possible that other microbial sensors, such as NOD2, are also involved in the regulation of the myeloid compartment. Based on these results we wondered whether mice with NOD2 knock-out (KO) would also have differences in the blood composition of myeloid cells. Unfortunately, we did not find differences in the number of myeloid subpopulations in the blood of these mice. However, human monocyte markers like CD14 or CD16 are not expressed in murine monocytes [136], which could partially explain the different results in the murine and human system. As it was not possible to transfer our data to the murine system, we continued to analyse human cells.

6.1.1. Role of NOD2 in myeloid cell differentiation

In addition to the finding that NOD2 SNP donors have less $CD33^+$ myeloid cells, a more detailed analysis of this population revealed an increased mean fluorescence intensity (MFI) of CD16 on monocytes isolated from blood collected with heparin tubes.

In humans, three subsets of monocytes have been identified based on the expression of CD14 and CD16 [137]. The subset of “classical” blood monocytes expresses CD14 (LPS co-receptor) and shows no expression of the low-affinity Fcγ-receptor CD16. This subset is the most abundant of the three and normally represents about 90% of all blood monocytes. The “intermediate” monocytes are characterized by a high expression of both CD14 and CD16. The “nonclassical” subset of monocytes is characterized by low expression of CD14 and high CD16 expression [137].

It has been suggested that the monocytes expressing different levels of CD14 and CD16 represent a continuum in monocyte differentiation [138]. Intermediate $CD14^+CD16^+$ monocytes derive from classical $CD14^+CD16^-$ and nonclassical $CD14^-CD16^+$ monocytes. Based on this, one can speculate that the observed increase in $CD14^+CD16^+$ monocytes is due to a block in cell differentiation, with more cells accumulating in this state.

$CD14^+CD16^+$ monocytes also show differences regarding their adhesion to the endothelium. This monocyte subpopulation adheres stronger compared with their $CD14^+CD16^-$ counterparts [139].

6.1.1.1. NOD2 status and dendritic cell differentiation/maturation

Based on our data with blood monocytes suggesting an impact of NOD2 SNPs on myeloid differentiation, we further analysed monocyte to DC and monocyte to macrophage differentiation. Although our analysis on mononuclear cell DC (MNC-DC) clearly demonstrates a defect in LPS-induced DC maturation and cytokine production,

other reports suggest that the presence of NOD2 polymorphisms only affect the phenotype of DCs stimulated with MDP but not LPS. However, in our hands MDP was not able to induce maturation of DCs independent of the NOD2 status. Nevertheless, other studies [137, 138, 139] used cells derived from Crohn´s disease patients, which is not comparable with our experiments as we used cells from healthy donors. Monocytes from Crohn's disease patients may be in a "pre-stimulated" state.

Contradictory to our findings in MNC-DC, DCs generated from monocytes (Mo-DCs) and stimulated with LPS expressed higher CD83 and CD86 levels upon maturation with LPS, indicating a stronger activation status of these cells. This finding is in line with a study from Sánchez-Schmitz G. et al [140]. In this study, the authors found that DCs generated from $CD16^+$ monocytes expressed higher levels of CD86, CD11a and CD11c, and showed lower expression of CD1a and CD32 compared to mo-DCs generated from $CD16^-$ monocytes. As we were able to demonstrate an increased amount of $CD16^+$ monocytes in NOD2 SNP-bearing donors, this population could be responsible for the high activation status of the generated mo-DCs. Furthermore, Ventura and colleagues [141] also found that DCs obtained from Crohn's disease patients with NOD2 mutations display an activated phenotype characterized by high CD86 expression.

6.1.1.2. NOD2 status and macrophage differentiation

NOD2 is also highly expressed in macrophages [142]. Although the majority of studies regarding NOD2 and antigen-presentig cells (APCs) make use of DCs, the effects of NOD2 SNPs in macrophages were also investigated.

In the present work few experiments were conducted studying monocyte to macrophage differentiation. Due to the big variation detected in the analysed macrophages, even from donors with the same NOD2 genotype, it is difficult to take conclusions regarding the impact of NOD2 status on macrophages differentiation. More donors need to be included in the analysis.

In a murine model, Murray et al [143] demonstrated that bone marrow-derived macrophages from NOD2-/- mice behaved in a similar way as macrophages from wildtype (WT) NOD2 mice, with the exception of extracellular-signal-regulated kinase (ERK) phosphorylation, that appeared to be slightly delayed when the cells were stimulated through toll-like receptor (TLR) 9 and TLR4. Nevertheless, cytokine production seemed to be unaffected. Other studies described that macrophages from Nod2-/- mice generated to express the disease-associated SNP13 Nod2 variant exhibit increased factor nuclear kappa B (NF-κB) activation and interleukin (IL)-1β secretion when stimulated with muramyldipeptide (MDP), suggesting that this common mutation acts as a gain-of-function mutation in this model [140]. However, unlike this Nod2-/- mouse

model, monocytes from patients or healthy individuals homozygous for the L1007insC mutation (SNP13) display a marked defect in NF-κB activation and IL-1β production in response to MDP [106].

6.1.2. Regulation of CD16

When analysing CD14/CD16 expression on elutriated human monocytes, we found that the difference in CD16 expression was no longer detectable. Further analyses revealed that CD16 expression was down-regulated on monocytes dependent on the cell preparation technique. Interestingly, the differences in CD16 expression between monocytes from WT donors and donors with SNP12 or SNP13 were again detectable after culturing the cells for 24 hours without stimulation indicating that NOD2 SNP12 or SNP13 play a role in the regulation of CD16 in human monocytes.

Upon TGF-β stimulation CD16 was up-regulated independent of the NOD2 status. Nevertheless, the highest number of $CD16^+$ cells was again detected in monocytes isolated from donors with NOD2 SNP12/13. In the course of inflammation TGF-β is often present in high amounts [141], and could induce this CD14/CD16 monocyte population especially in monocytes from donors with NOD2 SNP12 and SNP13. Platelets are an important source of TGF-β and are able to up-regulate CD16 [104, 142]. It is discussed that platelet-monocyte aggregates are present in human blood under physiological conditions [143]. This could also up-regulate CD16 expression especially in donors with NOD2 SNPs which seem to be more sensitive to the effects of TGF-β.

6.1.3. Role of NOD2 for monocyte activation

The intermediate population of $CD14/CD16^+$ monocytes has been identified as a major pro-inflammatory cell population [123]. They exhibit a distinct cytokine secretion pattern, with low IL-10 production and high levels of IL-1β, tumor necrosis factor (TNF) and IL-12. Moreover, it has been shown that these monocytes are more efficient antigen-presenting cells than its classical counterparts [144]. Based on these observations it has been proposed that intermediate monocytes play an important role in the pathogenesis of inflammatory disorders, such as in patients with active chronic disease [124]. Based on these data and our finding of an increased population of $CD14/CD16^+$ monocytes in donors with NOD2 SNPs, we studied cytokine section in monocytes with or without NOD2 SNPs. As expected, cytokine secretion was affected in relation to the NOD2 status.

No significant differences were found in IL-6, IL-1β and TNF secretion, but in line with the literature [106] a decrease in IL-8 secretion was detected in NOD2 SNP 12/13 donors compared with WT donors. IL-8 is known to be one of the most potent chemoattractant

molecules that, among several other functions, is responsible for guiding neutrophils through the tissue matrix until they reach sites of injury [145]. A decrease in IL-8 secretion could lead to a delayed bacterial clearance in case of infection, possibly due to a reduced number of neutrophils in the site of infection.

6.1.3.1. Impact of NOD2 on NF-κB signalling

After ligand binding, the NOD2 protein associates with a serine/threonine kinase called receptor-interacting protein 2 (RIP2) which in turn interacts with the regulatory subunit of the Iκκ complex. This leads to phosphorylation and degradation of IκBα and nuclear translocation of NF-κB. NF-κB is known to induce the expression of pro-inflammatory cytokines and other factors. Variants of the NOD2/CARD15 gene show an impaired activation of NF-κB *in vitro* in transfection experiments human embryonic kidney (HEK) 293T cells [146].

SNP13 is a frameshift mutation with the most drastic loss-of-function phenotype of all the three NOD2 SNPs. This SNP is normally associated with a markedly reduced NF-κB activation, while SNP8 and 12 normally respond better to bacterial stimulation than SNP13. Nevertheless, the ability to activate this pathway is still significantly reduced in all of the three variants [147].

Surprisingly, we detected a constitutive low Iκb level in the cells from a donor with combined NOD2 SNP 8+13 indicating increased activity rather than an incapability to activate NFκ-B. In line, donors with NOD2 SNP12 or SNP13 showed more spontaneous IL-6 production in non-stimulated monocytes compared with WT. Further analyses have to prove whether decreased IκB levels are also found in other SNP13 donors.

After bacterial stimulation with MDP or LPS, no difference was found in NF-κB activation between LPS and MDP, or between WT and SNP8 or SNP12. This result is in sharp contrast to data from transfection experiments with NOD2 SNPs [146]. Since we analysed only 3 donors our results have to be confirmed with more donor samples. However, it is possible that overexpression of mutated forms of NOD2 in tumor cell lines as done by Ogura et al. may not reflect the physiologic situation. Our donors have heterozygous mutations which mean that one WT allele is still present in the cell and this is the case in most published data on NOD2 SNPs in patient cells.

Based on the decreased IL-8 secretion in donor cells with NOD2 SNPs, one would expect a decrease in the activation of this signalling pathway. On the other hand, the secretion of IL-6, IL-1β and TNF was not altered in donors with NOD2 mutation. This indicates that the observed decrease in IL-8 secretion is not entirely dependent on the NF-κB pathway. NOD2 activation not only activates the NF-κB pathway but also mitogen-

activated protein (MAP) kinases such as ERK1/2 and c-Jun N-terminal kinases (JNK) [148], it would be interesting to determine these pathways in more detail in future studies.
Regarding Crohn´s disease or GvHD, it is discussed that NF-κB activation in epithelial cells is important for gut homeostasis [149]. Mice with an epithelial-specific deletion of Ikkβ showed a reduced expression of lymphopoietin and fail to develop a pathogen-specific Th2 response [150]. Further evidence also points to an important role of NF-κB for intestinal epithelial integrity and the interaction between the mucosal immune system and gut microflora: In a mouse model of epithelial cell-specific inhibition of NF-κB through a deletion of Ikk subunits essential for NF-κB activation, severe chronic intestinal inflammation occurred associated with increased apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides and translocation of bacteria into the mucosa [151]. Therefore, NOD2 mutations in epithelial cells rather than in myeloid cells could play an important role for Crohn´s disease and intestinal GvHD.

6.1.4. Role of NOD2 ligands for cell activation and differentiation

DCs are the most effective APCs in the activation of naïve T cells and therefore essential for the initiation of a primary immune response. They represent a bridge between innate and adaptive immunity [152].
Under steady state conditions DCs exist in an immature “tolerogenic” state. Upon microbial encounter they become activated and are now capable to stimulate T cells. The microbial products are normally recognized by TLRs and other microbial sensors [22], like the NOD-like sensors. Here, we compared the efficacy of possible NOD2 ligands such as MDP and LPS on DC maturation.
Although DCs express the NOD2 receptor, our analysis of the effect of MDP on Mo-derived DCs revealed that this molecule was unable to induce the maturation of DCs. Although several protocols were used for the generation of DCs, MDP was not able to successfully induce maturation. However, when the short time protocol using MNCs as a starting population was applied we detected a trend towards an increase of some of maturation-related markers, indicating that possibly lymphocytes are also targets of MDP stimulation and may support DC differentiation. There is evidence from the literature that NOD2 is also expressed and relevant in T cells. MDP stimulates NF-κB in human $FOXP3^+$ T cells and protects cells from apoptosis. This effect is not evident in T cells isolated from patients with NOD2 polymorphisms [153].
Since MDP failed to induce maturation, one hypothesis would be that MDP could actively induce a tolerogenic state in DCs. After analysing tolerogenic markers we only observed a trend towards an up-regulation of immunoglobulin-like transcript (ILT)3 and PD-L1, meaning that MDP is not able to induce a specific phenotype in DCs. Cytokine analysis

also showed no differences between immature DCs (iDCs) and DCs stimulated with MDP.

When a DC encounters bacterial stimulation, simultaneous pathways are activated through different pattern recognition receptors [154] . Although NOD2 stimulation alone was not able to induce phenotypical changes in DCs, it is possible that the sensing of this receptor is important in combination with other stimuli, such as TLR4.

Our data on cytokine production demonstrated an up-regulation on IL-10 secretion upon LPS and MDP combination and a decrease in IL-12 production, indicating a possible anti-inflammatory role of NOD2. These results are not completely in line with data found by Watanabe et al. [155] who pre-incubated DCs with MDP and observed a decrease in IL-12p40, IL-10 and IL-6 after stimulation with TLR ligands for all bacterial ligands compared to the response without pre-incubation. The synergistic role of TLRs and NOD2 seems not to be clear, since studies in mice demonstrated that NOD2 in combination with LPS was associated with an increased production of Th1 polarizing mediators [156].

A different study demonstrated that incubation of murine splenic macrophages with MDP suppressed IL-12p40 and IL-12p70 secretion induced by stimulation with TLR2 ligands, such as peptidoglycan [144]. This suppression was not observed in cells lacking NOD2 expression. *In vivo* experiments demonstrated similar results, since systemic administration of peptidoglycan to Nod2-/- mice induced more serum IL-12p40 and IL-12p70 compared to WT mice. Further analysis indicated that increased IL-12p70 production in peptidoglycan-stimulated NOD2-deficient cells was due to greater c-Rel-dependent *IL-12p35* mRNA expression. Taken together, these data suggest that inactivation of NOD2 enhances IL-12 secretion in murine macrophages due to the fact that NOD2 normally limits TLR2 agonist-stimulated expression of this cytokine.

Autophagy is a highly conserved self-degradation system that plays an important role in maintaining cellular homeostasis through the elimination of misfolded proteins and damaged organelles. Furthermore, autophagy is crucial for host defense against bacterial, viral, and parasitic pathogens [157]. In addition, it has been shown that autophagy has a major role in antigen presentation, with constitutive fusion of autophagosomes with multivesicular MHC class II-loading compartments in antigen-presenting cells. Simmons *et al.* [158] demonstrated that NOD2 induces autophagy in DCs that is required for NOD2-mediated antigen presentation and bacterial handling and is defective in the presence of NOD2 SNPs, demonstrating another mechanism by which DC function may be altered in donors bearing NOD2 SNPs.

6.2. Role of vitamin D_3 on immune regulation

There is accumulating evidence that vitamin D_3 signalling plays a role in the regulation of the immune system from several studies [79]. The vitamin D receptor (VDR) is expressed in most cells of the immune system, including T cells, neutrophils and APCs such as macrophages and DCs [159].

The main vitamin D metabolite in serum is $25(OH)D_3$ and it is converted to the active form 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) by Cytochrome P450 Family 27 Subfamily B Member 1 (CYP27B1). This enzyme is expressed in the kidney but also by activated myeloid immune cells. The active form binds to the VDR and regulates several genes e.g. the induction of antimicrobial peptides such as cathelicidin and β-defensin. These antimicrobial peptides play an important role in clearing infections since they inhibit the growth and kill bacteria. In addition, defensins also amplify adaptive immune responses resulting in both Th1- and Th2-dependent responses by activation of immature DCs [80].

It is also known that $1,25(OH)_2D_3$ regulates the expression of pattern recognition receptors (PRRs). It has been shown that $1,25(OH)_2D_3$ has the ability repress TLRs and inhibits the secretion of pro-inflammatory cytokines [160]. Moreover, Wang et al. showed that vitamin D_3 has the capability to induce NOD2 expression [161]. However, in the present work only a slightly induced expression of *NOD2* mRNA was detected upon incubation of monocyte with $1,25(OH)_2D_3$. This difference may be due to the fact that Wang. et al. used 100 nM of $1,25(OH)_2D_3$, which is a high, non-physiological concentration. Given the importance of NOD2 as a signal for the regulation of inflammation, it seems plausible that vitamin D_3 acts synergistically with the NOD2 receptor helping to suppress inflammation.

Furthermore, $1,25(OH)_2D_3$ is capable of inducing tolerogenic DCs [111]. In line, we were able to demonstrate that $1,25(OH)_2D_3$ induces *ILT3* expression, a known biomarker of tolerogenic DCs [111].

It is known that the active vitamin D metabolite $1,25(OH)_2D_3$ has not only strong effects on the generation of tolerogenic DCs but also inhibits T cell function [29, 111]. Here, we investigated whether both vitamin D_3 forms, 25-hydroxyvitamin D3 ($25(OH)D_3$) and $1,25(OH)_2D_3$, also have direct effects on T cells, independent of DC presence. This would implicate that T cells metabolize $25(OH)D_3$ to $1,25(OH)_2D_3$ on their own.

6.2.1. Effect of vitamin D_3 on human T cells

In the present study, the direct effects of the vitamin D_3 precursor, $25(OH)D_3$ or the active form of vitamin D_3 ($1,25(OH)_2D_3$) on $CD4^+$ T cells was evaluated.

In humans, serum levels of $25(OH)D_3$ in a range of 50-100 nM have been defined as sufficient levels [162]. Therefore, in the first set of experiments, T cells were incubated with a physiological concentration of 50 nM $25(OH)D_3$ and stimulated with different T cell expander bead (TCE) bead: cell ratios. Since it is known that tolerogenic dendritic cells have a weaker ability to stimulate T cells, we hypothesised that a weaker stimulation level with lower amounts of beads would give rise to higher amounts of regulatory T cells. Although this was not the case, $25(OH)D_3$ was able to induce FOXP3 expression independent of the used bead concentration.

In this first approach, $CD4^+$ T cells isolated from blood were used and this mixed population is composed of $CD4^+CD25^+$, which include circulating $FOXP3^+$ cells, and naïve $CD4^+CD25^-$ cells. Therefore, it is difficult to distinguish whether $25(OH)D_3$ expands pre-existing $FOXP3^+$ cells or induces $FOXP3^+$ cells from $CD4^+CD25^-$ naïve T cells. To overcome this, naïve $CD4^+CD25^-$ T cells were sorted and used to study the impact of $25(OH)D_3$ and $1,25(OH)_2D$ on T cells. After flow cytometry analysis, we were able to demonstrate that physiological concentrations of both $25(OH)D_3$ and $1,25(OH)_2D_3$ were able to up-regulate FOXP3 expression in naïve T cells. These results prove that $25(OH)D_3$ and not only $1,25(OH)_2D_3$ directly modulates T cells.

There are already some studies showing an effect of $1,25(OH)_2D_3$ on Tregs [118, 163], but these studies use high non-physiological concentrations of $1,25(OH)_2D_3$. One study compared the effects of $25(OH)D_3$ and $1,25(OH)_2D_3$ on human T cells and stated that $25(OH)D_3$ is not capable to directly act on T cells. The authors state that $25(OH)D_3$ has to be converted to $1,25(OH)_2D_3$ through the action of DCs [116], since it is known that APCs are capable of converting the circulation form of $25(OH)D_3$ to its active form. In addition, it has also been shown that $1,25(OH)_2D_3$ induces a tolerogenic phenotype in DCs, decreasing IL-12 secretion and increasing IL-10 [111], eventually leading to Treg induction. Nevertheless, since T cells also express the VDR and CYP27B1 [164, 165] it seems plausible that $25(OH)D_3$ can be converted to $1,25(OH)_2D_3$ which then has a direct effect on T cells. In line with this, we were able to prove that T cells can actively convert $25(OH)D_3$ to its active form $1,25(OH)_2D_3$. On the other hand, 25(OH)D3 has been reported to bind with low affinity to VDR [166]. Therefore, it remains to be elucidated whether the effect of $25(OH)D_3$ on T cells is a result of its conversion to the active vitamin D metabolite and subsequent action of $1,25(OH)_2D_3$ on the cells or whether $25(OH)D_3$ exerts direct effects on T cells.

Since it is known that TGF-β is a potent inducer of Tregs [117], we included TGF-β in our experiments as a control. In addition, we aimed to study its possible synergy with $25(OH)D_3$ and $1,25(OH)_2D_3$. The results from the present work demonstrate that the addition of both forms of vitamin D_3 to TGF-β did not further increase the number of $FOXP3^+$ cells analysed by flow cytometry, although we detected a significant increase of *FOXP3* mRNA in the combined use of TGF-β and $1,25(OH)_2D_3$. In further analyses we investigated the functional impact of $25(OH)D_3$ and $1,25(OH)_2D_3$ on Tregs.

Cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) is a suppressive protein that is expressed constitutively by Tregs and is induced on conventional T cells following activation. Its main function is to restrain inappropriate activation of autoreactive T cells and to restore T cell homeostasis following activation [167]. We detected an increase in the alternatively spliced soluble CTLA-4 (sCTLA4) isoform in the culture supernatant of T cells in the presence of 1,25(OH)D3 and the highest increase was found in cultures stimulated with both TGF-β and 1,25(OH)2D3. In line with this finding, we also found an increased IL-10 production in cultures stimulated with a combination of $1,25(OH)_2D_3$ and TGF-β, demonstrating an additive effect of these both agents.

IL-17 is a strong inducer of inflammation and has an important role in protecting the body against invading pathogens. It is mainly produced by Th17 cells and this population needs to be kept in a strict balance, since it contributes to tissue damage in inflammation such as gut GvHD [168]. For this reason, we also measured IL-17 in the culture supernatants and found that $1,25(OH)_2D_3$ treatment decreased the secretion of this cytokine, demonstrating a possible role for vitamin D_3 in the attenuation of Th17 responses.

Finally, reduced IFN-γ secretion was found upon $1,25(OH)_2D_3$ addition to the cultures. IFN-γ is a classical Th1 cytokine, so this result is in line with the literature that states that $1,25(OH)_2D_3$ inhibits Th1 responses, favouring Th2 [80].

Taken together, the data on vitamin D_3 and T cells clearly demonstrates that even physiological amounts of both $25(OH)D_3$ and $1,25(OH)_2D_3$ are able to induce a regulatory phenotype in T cells. Although an increase in the percentage of $FOXP3^+$ cells was not detected in the combination of $1,25(OH)_2D_3$ and TGF-β, analysis of the culture supernatants demonstrated a clear additive effect of both stimuli on extracellular levels of sCTLA4, IL17 and IFN. As the cytokine profile is an important mediator of the immune response, combination of TGF-β and vitamin D_3 could balance anti- and pro-inflammatory cytokines, necessary for effective tolerance induction. The protective role of $25(OH)D_3$ has already been demonstrated in several diseases. Namely, vitamin D_3 supplementation was observed to reduce disease activity in patients with CD, as well ameliorated experimental models of inflammatory bowel disease (IBD) [169]. Also, vitamin D_3 levels

have been related with the outcome of hematopoietic stem cell transplantation (HSCT) [89]. This could in part be due to effects on Treg induction since in humans Prietl et al. demonstrated an association of vitamin D_3 supplementation with an increase in $CD4^+Foxp3^+$ T cells in circulation [170, 171].

In a different setting, Hjelte et al. studied the impact of vitamin D_3 supplementation in Cystic fibrosis patients. This disease is characterized by a persistent lung inflammation [172] and the authors found that total $25(OH)D_3$ dose/kg bodyweight correlated with the down-regulation of CD86 on mDCs. Furthermore, they reported a reduced programmed cell death protein 1 (PD-1) expression on CD4 and CD8 cells, as well as a decreased frequency of $CD8^+$ T cells co-expressing the activation markers CD38 and HLA-DR in a dose-dependent fashion.

The impact of vitamin D_3 on human monocytes was also investigated *in vivo* in donors infected with human immunodeficiency virus HIV. Sereti et al. [173] found that patients with vitamin D_3 deficiency, when compared to those with insufficient or normal vitamin D_3 levels had increased levels of IL-6, TNF-α and higher proportions of $CD14^{dim}CD16^+$ and $CX_3CR_1^+$monocytes. A decreased frequency of $CCR2^+$ monocytes was also detected in this study. In summary, in line with our data, in vivo $25(OH)D_3$ levels also seem to be important for proper immune regulation as vitamin D_3 deficiency is often associated with more prone inflammatory conditions.

6.2.2. *In vivo* effects of vitamin D_3 on mice

The *in vivo* effects of vitamin D_3 have also been studied in different mouse models such as VDR-KO or CYP27B1-KO models which demonstrated that defects in the vitamin D metabolism are associated with an inhibition of T cell proliferation, inhibition of IFN-γ, IL-17 and induction of IL-4 [88].

Koeffler and colleagues [84] demonstrated that VDR-KO mice have normal numbers of T and B lymphocytes, but differences in their cytokine responses. The authors found that splenocytes from VDR-KO mice secreted less IFNγ and more IL-4 compared with splenocytes from WT mice. Moreover, VDR expression seems to be crucial for the appropriate development of invariant natural killer (iNKT) cells, a subset of lymphoid cells involved in basic immune responses and also restricting autoimmunity [85]. Cantorna et al. noted a reduced number of these cells in a VDR-KO mouse model.

In a model of IBD it was also demonstrated that VDR-KO mice had reduced numbers of CD8αα intraepithelial lymphocytes (IEL). In addition, low IL-10 production in the IEL and the failure of the VDR-KO T cells to home to the small intestine was detected.

Besides its impact on T cells, the VDR also influences the number of myeloid cells. Song et al. [174] showed that the number of macrophages is markedly reduced in wounds of

vitamin D_3-deficient mice and mice with macrophage-specific VDR deletion, demonstrating that the expression of the VDR in macrophages is essential for the normal expansion of tissue-resident macrophages.

In this thesis we used no VDR-KO mouse model but the more physiological setting of different vitamin levels in mice to study the impact of vitamin D levels on immune cell composition. Besides blood immune cells we also investigated immune cells in the spleen and bone marrow as important sites of immune cell differentiation. Three independent experiments, using two different animal models were performed: BALB/c, an inbred mouse strain and CD-1, an outbred strain. In two experiments BALB/c mice were used. The animals were the same age, in the same animal facility and were purchased from the same company. Nevertheless we found great variations between both vitamin D_3 levels and immune cell composition that were not expected. Even though this animal model should provide solid results due to genetic similarities owed to long inbreeding, results indicate that yet unidentified variables modulate the immune system of these mice. Surprisingly, the outbred strain CD-1 mice showed no significant differences to the used BALB/c mice. In the main lymphoid blood cell populations such as B and T cells, no significant effects of vitamin D_3 were detected in BALB/c mice, with the exception of experiment 2 using BALB/c mice where more $TCR\beta^+$ cells were detected in the bone marrow of animals fed with sufficient levels of vitamin D_3. This result was also observed in the bone marrow of outbred strain CD-1 and contradicts in part the VDR-KO data models were no differences were found in the total number of these cells [84].

Furthermore, CD-1 mice fed with sufficient vitamin D_3 seem to have less $CD19^+$ B cells in their spleen compared to animals having a deficient vitamin D_3 diet.

When analysing myeloid cell subpopulations, we detected in two experiments an increase in myeloid-derived suppressor cells (MDSC) in animals with sufficient levels of vitamin D_3 in blood and spleen.

MDSC have been shown to ameliorate GvHD [175] and to mitigate dextran sulfate sodium (DSS)-induced colitis [176]. The finding that $25(OH)D_3$ also positively modulates this cell population provides another possible mechanism by which vitamin D_3 could be beneficial in an inflammatory setting. Furthermore, using a murine IBD model Qin et al. [177] made use of the combined properties of MDSC and vitamin D_3 in a sophisticated monocyte-based adoptive CYP27B1 gene therapy approach where they used the traffic capacity of MDSC to inflamed colon to deliver local amounts of vitamin D_3. In this system the authors used a targeting strategy with $CD11b^+/Gr1^+$ monocytes as the cell vehicle and a macrophage-specific promoter (Mac1) to control CYP27B1 expression. Local amounts of vitamin D_3 modulated T cells (switch from Th1 to Th2), cytokine secretion and promoted mucosal regeneration via epithelial tight junction protein synthesis.

Although we expected an increased amount of $CD4^+FOXP3^+$ cells in mice with sufficient levels of $25(OH)D_3$ compared with deficient ones, we were not able to prove this finding in our experimental setting. Possibly, Tregs are only modulated by vitamin D under inflammatory conditions where TGF-β is present and not under steady state conditions, since it is known that TGF-β is a positive regulator of Tregs and we proved a synergistic effect of vitamin D_3 and TGF-β *in vitro*. Therefore, in mice with sufficient vitamin D_3 levels, when facing inflammation, vitamin D_3 could synergize with TGF-β and other cytokines, increasing the number of Tregs. Nevertheless, in one experiment we detected that Helios, a transcription factor expressed in thymus-derived Tregs, [122]could also be a target for vitamin D_3, as more cells expressing Helios were detected in the bone marrow of animals fed with sufficient vitamin D_3.

6.3. Impact of vitamin D_3 on microbiota

Several mechanisms have been proposed through which vitamin D_3 may influence the gut microbiota. Vitamin D_3 can affect both immune cells and colonic epithelial cells; modulating colon barrier function, and the secretion of antimicrobial peptides, mucins, and cytokines, all of which have the potential to modulate gut bacteria [178].

In a recent study from Franke et al. [179] the authors identified that variations in the VDR influenced the gut microbiome. Additionally, they provided compelling follow-up to the finding of Makishima *et al*. that demonstrated that secondary bile acids (which are produced by special bacteria in the gut) serve as ligands for VDR.

Furthermore, it has been demonstrated that vitamin D_3-deficient mice have decreased expression of angiogenin-4 (an antimicrobial peptide) within the colon and consequently have an increased bacterial load with decreases in Lactobacillus species and increases in *Clostridium* and *Bacteroides* species within the colon, suggesting that vitamin D_3 deficiency may impair microbial homeostasis [180]. Furthermore, mice lacking the ability to respond to vitamin D_3 (either due to lack of VDR or CYP27B1) have differences in their gut microbiome compared to WT littermates with intact vitamin D_3 signalling [181] demonstrating that host responses to vitamin D_3 impact the microbiome. In this study, Cantorna and co-workers found that Firmicutes and Bacteroidetes were the dominant phyla in all mice (KO or WT). Bacteroidetes, Proteobacteria, and unclassified bacteria were over-represented in the fecal microflora of Cyp-KO and VDR-KO mice relative to their WT counterparts. Equally, the Cyp-KO and VDR-KO mice had fewer bacteria from the Firmicutes and Deferribacteres phyla compared with their respective WT littermates.

In our analyses, we detected higher bacterial diversity in mice with deficient vitamin D_3 compared with mice with vitamin D_3 sufficiency. Similarly to findings of Cantorna et al., the most dominant phyla detected in vitamin D-deficient mice were Bacteroidetes.

However, an increased amount of this phylum as well as Firmicutes was detected in mice with vitamin D_3 deficiency. On the other hand, increased amounts of Verrocomicobia were detected in mice with sufficient vitamin D_3 levels.

Akkermansia was the only bacteria genus found in significantly greater amount in the stool of vitamin D_3 sufficient animals. These results are in line with findings by Han and colleagues [182] who observed a marked reduction in *Akkermansia* in VDR-KO mice. Although there are 8 identified species belonging to this genus, most reports concern *Akkermansia muciniphila.* These mucin-degrading bacteria have been reported to be in low numbers in patients with IBD [183] compared with healthy individuals, have been identified as capable Treg inducers, have proven to be beneficial in colitis and shown to induce the expression of antimicrobial peptide Reg3γ in the colon [184]. Taken together, the results prove a strong relationship between vitamin D_3 status and gut microbiota, and how these two factors could shape the immune response.

6.4. Perspectives

The importance of the NOD2 status has been acknowledged for some years for several inflammatory conditions such as CD and HSCT outcome. Although the possible role of NOD2 has been established as a suppressor of inflammation, the exact mechanism by which this occurs is not clear. In the present work, we found a decreased amount of myeloid cells in healthy donors bearing the studied NOD2 mutations, indicating a possible role for NOD2 in haematopoiesis. Furthermore, an increased CD16 expression was detected in NOD2 mutants, which can be related with an increase activation of myeloid cells, characteristic of inflammatory conditions. Since we also demonstrated the capacity of platelets and TGF-β in upregulating CD16, the amount of TGF-β as well as the presence of monocyte-platelet aggregates in circulation should be investigated in donors bearing the NOD2 mutations in comparison with WT individuals.

Since neutrophils represent the main myeloid blood cell population, in further studies it would be interesting to analyse neutrophils counts in blood.

The signalling pathways involved with NOD2 activation, such as NF-κB and phospho p38 also seem to be involved in the NOD2 effects and should be analysed in more detail. Although we detected a higher IL-6 secretion in non-stimulated NOD2 SNP-bearing donors, we were only able to detect what seems to be constitutive activation of NF-κB in one donor, harbouring a SNP8 and a SNP13, meaning that the presence of both mutations could constitute a special phenotype. Nevertheless, more donors should be included in the analysis. Data on cell differentiation/maturation suggested and increased activation of DCs generated from NOD2 mutated donors compared with WT, what goes

in line with the CD16 findings. More donors should be included and the T cell stimulatory capacity of these cells should also be investigated.

Regarding the *in-vitro* T cells studies, we proved that both $25(OH)D_3$ and $1,25(OH)_2D_3$ are capable of directly modulate the T cell phenotype, in an additive fashion with TGF-β. Although TGF-β is able to induce FOXP3 expression, it is known that this expression is not stable. Stable Tregs are characterized by the demethylation of a CpG-rich element within the FOXP3 locus, so naturally the next step on evaluating the vitamin D_3 effect on T regs would be analysing the methylation status of the *FOXP3* gene after vitamin D_3 treatment. To access the stability of the phenotype, the cells should be cultured in a first period in the presence of vitamin D_3 and/or TGF-β and in a second period this stimulus should be removed from culture. After this period the cell phenotype should be investigated in order to compare with the initial culture period.

Although due to experimental variability no clear effects were found in the *in vivo* studies regarding vitamin D_3, the most interesting finding is possibly the significant relation between vitamin D_3 status and MDSC.

Due to the findings relating vitamin D_3 and inflammatory conditions such as IBD and GvHD, further studies need to be conducted in order to clarify the exact mechanisms leading to these observations. After establishment of vitamin D_3-sufficient and -deficient mice and analysing their respective microbiota, bone marrow transplantation of these animals should be performed. Since the effects of vitamin D_3 are vast and not restricted to one cell population and cover several aspects of the host immune response, two approaches can be followed. The first one would be to analyse the impact of the vitamin D_3 in the host. In this way, one would expect that the vitamin D_3 effects were due to its impact on intestinal epithelia (main target of GvHD) and microbiota modulation by vitamin D_3.

The second approach would be to study the impact of vitamin D_3 on immune cells, and for this, bone marrow and spleen cells of vitamin D_3-sufficient or -deficient donor mice should be injected in irradiated recipients. Although it is known that there are several cells, especially APCs, resistant to irradiation the mice are then immune-reconstituted with cells with different vitamin D_3 backgrounds (sufficient and deficient). This would be probably the most accurate approach to study the impact of vitamin D on immune cells and the outcome of bone marrow transplantation.

In conclusion, this work demonstrates a new mechanism by which NOD2 mutations could lead to a more inflammatory-prone condition, by the increased number of $CD16^+$ monocytes, deregulated NF-κB pathway and differences in dendritic cell maturation/activation. Furthermore we demonstrate a synergistic effect of vitamin D_3 and TGF-β for the generation of regulatory T cells. The *in vivo* effects of vitamin D_3

demonstrated a positive relation between vitamin D_3 and MDSC and the modulation of the murine microbiome, with a significant loss of Akkermansia in mice with vitamin D_3 deficiency. A more detailed study on the impact of the gut microbiota and vitamin D_3 should be performed, studying the expression of proteins involved in maintaining the epithelial barrier function.

7. Summary

GvHD is still the most common life-threatening complication associated with allogeneic hematopoietic stem cell transplantation (HSCT). NOD2/CARD15, an intra-cytoplasmatic pathogen-recognition receptor recognizing bacterial muramyl dipeptide (MDP), has gained substantial interest, as single nucleotide polymorphisms (SNPs) of this receptor have been identified as a risk factor for Crohn´s disease and GvHD. These data suggest that MDP sensing by hematopoietic cells is of crucial importance for the immune homeostasis in target organs of GvHD, especially in the gastrointestinal tract and/or skin. Nevertheless, the exact mechanism by which the mutation in the NOD2 receptor affects the outcome of transplantation is not entirely understood.

In the present work, the impact of NOD2 polymorphisms on the immune cell composition in peripheral blood of healthy donors was evaluated. We found that donors with NOD2 mutations presented differences in their myeloid compartment, with less $CD33^+$ cells (a common myeloid marker) and less myeloid dendritic cells (mDCs). A more detailed analysis on the monocyte population revealed that donors with SNP12 or SNP13 but not SNP8 had increased CD16 expression. $CD14^+CD16^+$ monocytes have been identified as a major proinflammatory cell population as they exhibit a distinct cytokine secretion pattern, with low IL-10 production and high levels of IL-1β, TNF and IL-12. Moreover, it has been shown that these monocytes are more efficient antigen-presenting cells than their classical counterparts. These results provide a new insight by which NOD2 polymorphisms can have a modulatory function in the course of inflammation.

TGF-β is a pleiotropic cytokine with strong regulatory and inflammatory activity. Our analysis demonstrated that TGF-β is capable of inducing the expression of CD16 on monocytes, independent of the NOD2 status. However, we noticed that the highest number of $CD14^+CD16^+$ cells was found in donors with SNP12 or 13 mutations. The cytokine profile of these cells revealed a decrease in IL-8 secretion by SNP12 or 13 donors and, although not significant, a strong trend towards an increased IL-6 secretion, especially under non-stimulatory conditions. In line with these observations, we analysed the expression of iκB and found a constitutive degradation of IκB in one donor with both SNP8 and SNP13 mutation, indicating a constitutive activation of the NF-κB pathway.

The imbalance found in the immune cell composition of the analysed NOD2 SNP donors could contribute to the association seen between NOD2 SNPs and HSCT outcome. The cellular imbalances reflect at molecular level through pro-inflammatory cytokine secretion, and it is believed that a dysregulation on cytokine production provide the first mechanism by which NOD2 variants can affect the outcome of HSCT.

Beside NOD2 polymorphisms, vitamin D_3 receptor gene polymorphisms have also been associated with GvHD. Furthermore, it has already been shown that vitamin D_3 deficiency is a common phenomenon in allogeneic transplant patients and may also be involved in the pathogenesis of Crohn's disease and GvHD. The impact of 1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) on T cells is well documented. It is known that this vitamin is capable of inducing FOXP3 expression, a known marker for regulatory T cells. However, the majority of the studies make use of high, non-physiological amounts of $1,25(OH)_2D_3$. Furthermore, the impact of vitamin D_3 on T cells is commonly mediated through the action of dendritic cells. In addition, the impact of vitamin D_3 precursor, $25(OH)D_3$ is poorly documented.

In the present work, we demonstrated that T cells are direct targets of vitamin D_3. Both $25(OH)D_3$ and $1,25\ (OH)_2D_3$ were able to upregulate FOXP3 expression on human $CD4^+$ T cells, independent of dendritic cells. Also, we demonstrated a synergistic effect of vitamin D_3 and TGF-β in the induction of a regulatory phenotype on T cells. Although the expression of FOXP3 was not different in cells treated with TGF-β and $1,25(OH)_2D_3$, compared with TGF-β alone, cytokine analysis demonstrated an increased IL-10 and sCTLA4 in the presence of both stimuli. Furthermore, both $25(OH)D_3$ and $1,25(OH)_2D_3$ showed a strong trend in the reduction of IL-17 and IFNγ secretion.

The use of a vitamin D_3 sufficient vs deficient mice model proved to be rather inconsistent between the three performed experiments. However, a significant increase in the number of myeloid-derived suppressor cells in mice with sufficient levels of vitamin D_3 was found. Since MDSC were found to ameliorate GvHD and DSS- induced colitis, this finding supports the relations found between vitamin D_3 status and inflammatory conditions.

The impact of vitamin D_3 on microbiome was also analysed and an increased bacterial diversity on mice with low levels of vitamin D_3 was detected. Furthermore, a significant increase in bacteria from the *Akkermansia* genus was found in the stool of mice with sufficient levels of vitamin D_3. These bacteria are known to be capable Treg inducers, have proven to be beneficial in colitis and shown to induce the expression of antimicrobial peptide Reg3γ in the colon. These data provide further proof on the importance of vitamin D_3 status on the resolution of inflammatory conditions.

8. Zusammenfassung

Die GvHD gilt nach wie vor als die häufigste, lebensbedrohliche Komplikation, die bei einer allogenen hämatopoetischen Stammzelltransplantation (HSZT) auftreten kann. NOD2/CARD15, ein pathogen-spezifischer Rezeptor im Zytoplasma, der das bakterielle Muramyl-Dipeptid (MDP) erkennt, hat zunehmend an Bedeutung gewonnen, da Einzelnukleotid-Polymorphismen (SNPs) dieses Rezeptors als Risikofaktor für Morbus Crohn und die GvHD gelten. Diese Erkenntnis deutet darauf hin, dass das Erkennen von MDP durch hämatopoetische Zellen für die Immunhomeostase in Zielorganen der GvHD, wie dem Magen-Darm Trakt und/ oder der Haut, von großer Bedeutung ist. Allerdings ist der exakte Mechanismus, auf welche Weise die Mutation im NOD2 Rezeptor den Ausgang einer Transplantation beeinflusst, nicht vollständig geklärt.

In dieser Arbeit wurde deshalb untersucht, wie sich NOD2 Polymorphismen auf die Immunzellkomposition im peripheren Blut gesunder Spender auswirken. Spender mit einer NOD2 Mutation wiesen Unterschiede in der Zusammensetzung der myeloiden Zellen auf, wobei weniger $CD33^+$ Zellen (ein klassischer myeloider Marker) und weniger myeloide dendritische Zellen (mDCs) detektiert wurden. Genauere Untersuchungen von Spendern mit einer SNP12 oder SNP13, nicht aber mit einer SNP8 Mutation, zeigten eine erhöhte CD16 Expression in Monozyten. Aufgrund ihres unterschiedlichen Zytokinprofils mit einer geringen IL-10, sowie eine hohe IL-1β, TNF und IL-12 Sekretion, gelten $CD14^+CD16^+$ Monozyten als die wichtigste proinflammatorische Zellpopulation. Es konnte gezeigt werden, dass diese Monozyten im Vergleich zu den klassischen Monozyten durch eine effizientere Antigenpräsentation charakterisiert sind. Diese Ergebnisse liefern neue Anhaltspunkte, inwieweit NOD2 Polymorphismen modulatorisch bei dem Ablauf einer Entzündungsreaktion wirken könnten.

TGF-β ist ein pleiotropes Zytokin mit stark regulatorischen und inflammatorischen Eigenschaften. Analysen im Rahmen dieser Arbeit zeigten, dass TGF-β, unabhängig vom NOD2 Status, die CD16 Expression auf Monozyten induzieren kann. In Spendern mit einer SNP12 oder SNP13 Mutation war der Effekt jedoch am stärksten und es wurden die meisten $CD14^+CD16^+$ Zellen detektiert. Das Zytokinprofil von Monozyten zeigte eine Reduktion der IL-8 Sekretion in SNP12 oder SNP13 mutierten Spendern und, obwohl nicht signifikant, vor allem unter nicht stimulierten Bedingungen trendmäßig eine erhöhte IL-6 Sekretion. Aufgrund dieser Beobachtung analysierten wir die Expression von iκB und fanden eine starke Reduktion von iκB in einem Spender mit gleichzeitiger SNP8 und SNP13 Mutation, was auf eine dauerhafte Aktivierung des NF-κB Signalwegs hinweist.

Die unterschiedliche Immunzellkomposition in den analysierten NOD2 SNP Spendern könnte den beobachteten Zusammenhang zwischen den NOD2 SNPs und dem Ausgang einer HSZT erklären, da eine Dysregulation der Zytokinproduktion den ersten Schritt darstellt, durch den NOD2-Varianten das Ergebnis einer HSZT beeinflussen könnten.

Neben dem NOD2 Polymorphismus, wird der Vitamin D_3 Rezeptor Polymorphismus ebenfalls mit der GvHD in Verbindung gebracht. Es wurde bereits gezeigt, dass ein niedriger Vitamin D_3 Spiegel in allogen transplantierten Patienten vorherrscht und dass dieser eine Rolle in der Pathogenität von Morbus Crohn und der GvHD spielen kann.

Der Einfluss von 1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) auf T Zellen wurde in der Literatur bereits beschrieben. Es wurde gezeigt, dass dieses Vitamin die Expression von FOXP3 induzieren kann, ein klassischer Marker für regulatorische T Zellen. Jedoch wurde in einem Großteil der Studien eine sehr hohe, nicht physiologische Konzentration an $1,25(OH)_2D_3$ eingesetzt. Zudem wird der Einfluss von Vitamin D_3 auf T Zellen oftmals indirekt durch die Interaktion mit dendritischen Zellen erklärt. Über den Effekt von $25(OH)D_3$, dem Vorläufermolekül von Vitamin D3, ist nur wenig bekannt.

In dieser Arbeit konnte gezeigt werden, dass Vitamin D_3 T Zellen direkt beeinflusst. Sowohl $25(OH)D_3$ als auch $1,25(OH)_2D3$ waren in der Lage die FOXP3 Expression in humanen $CD4^+$ T Zellen zu steigern, unabhängig von der Stimulation durch dendritische Zellen. Zudem zeigten wir einen synergistischen Effekt zwischen Vitamin D_3 und TGF-β, der zur Ausprägung des regulatorischen Phänotyps in T Zellen führte. Obwohl kein Unterschied in der FOXP3 Expression in TGF-β und $1,25(OH)_2D_3$ behandelten Zellen im Vergleich zu einer Behandlung ausschließlich mit TGF-β bestand, ergab die Untersuchung der Zytokine eine erhöhte IL-10 und sCTLA4 Sekretion unter Stimulation mit beiden Substanzen. Sowohl $25(OH)D_3$ als auch $1,25(OH)_2D_3$ führten trendmäßig zu einer Reduktion der IL-17 und IFNγ Sekretion.

Drei Experimente in einem Mausmodell, bei dem eine Diät mit ausreichend viel gegenüber zu wenig Vitamin D_3 gefüttert wurde, lieferten widersprüchliche Ergebnisse. Allerdings nahm die Anzahl der myeloiden Suppressorzellen (MDSC) in Mäusen mit ausreichendem Vitamin D3 Spiegel zu. Da MDSCs die Ausprägung einer GvHD und eine DSS-induzierte Kolitis abschwächen können, deuten unsere Ergebnisse auf einen Zusammenhang zwischen dem Vitamin D_3 Status und der Schwere einer Entzündung hin.

Bei der Analyse des Effekts von Vitamin D_3 auf das Mikrobiom wurde eine erhöhte bakterielle Diversität in Mäusen mit geringem Vitamin D_3 Spiegel beobachtet. Außerdem wurden signifikant mehr Bakterien der Gattung Akkermansia im Stuhl von Mäusen mit ausreichendem Vitamin D_3 Spiegel detektiert. Diese Bakterien können die Differenzierung von regulatorischen T Zellen auslösen, begünstigen den Ausgang einer

Kolitis und steigern die Expression des antimikrobiellen Reg3γ-Peptid im Kolon. Diese Ergebnisse deuten auf eine wichtige Rolle des Vitamin D_3 Status für die Regulation einer Entzündung hin.

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Publications

Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer 1, Thiel A, **Matos C,** (…), Kreutz M. LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. Cell Metabolism 2016 Nov 8;24(5):657-671

Tobias Roider, Michael Katzfuß, **Carina Matos**, Katrin Singer, Kathrin Renner, Peter Oefner, Katja Dettmer-Wilde, Wolfgang Herr, Ernst Holler, Marina Kreutz, Katrin Peter. Antithymocyte Globulin Induces a Tolerogenic Phenotype in Human Dendritic Cells. International Journal of Molecular Sciences 2016

Sakhila Ghimire, **Carina Matos**, Massimiliano Caioni, Daniela Weber, Katrin Peter, Ernst Holler, Marina Kreutz, Kathrin Renner, Indoxyl 3-Sulfate Inhibits Maturation and Activation of Human Monocyte-derived Dendritic Cells Immunobiology 2017

Katrin Peter, Peter J. Siska, Tobias Roider, **Carina Matos**, Kathrin Renner, Katrin Singer, Wolfgang Herr, Ernst Holler, Klaus Stark, Iris Heid, Marina Kreutz, High 1,25-dihydroxyvitamin-D3 levels improve outcome during allogeneic hematopoietic stem cell transplantation, Blood, 2017 *submitted*

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