Inflammatory Bowel Disease and Dendritic Cells: 
A Proteome Analysis of Dendritic Cell Effector States

Inaugural-Dissertation 
zur Erlangung des Doktorgrades 
der Medizin

der Medizinischen Fakultät 
der Eberhard Karls Universität 
zu Tübingen

vorgelegt von

Popov, Todor

2017
Dekan: Professor Dr. I. B. Autenrieth

1. Berichterstatter: Professor Dr. J.-S. Frick
2. Berichterstatter: Professor Dr. N. Malek

Tag der Disputation: 25.07.2016
For my family
# Table of Contents

1 Introduction .............................................................................. 1

1.1 Inflammatory bowel disease (IBD) ................................. 1

1.2 Microbiota, *E. coli* mpk2 and *B. vulgatus* mpk ...... 3

1.3 Dendritic cells and their role in IBD. ......................... 6

1.4 Reactive oxygen species .............................................. 17

1.5 ROS and “leaky gut” syndrome ................................. 19

1.6 Antioxidants, Nrf2-pathway and Prohibitin

(Phb1). .................................................................................. 20

1.7 Aims ............................................................................. 25

2 Materials and Methods ......................................................... 27

2.1 Bacterial cultures .......................................................... 27

2.1.1 *Escherichia coli* mpk2 ............................................. 27

2.1.2 *Bacteroides vulgatus* mpk ..................................... 27

2.2 Cell culture ................................................................. 28

2.2.1 Bone marrow derived murine

  dendritic cells (BMDCs). ............................................... 28

  2.2.1.1 Cell culture medium for BMDCs ...................... 28

  2.2.1.2 Isolation and culturing of BMDCs ............... 28

2.2.2 *In vitro* dendritic cell stimulation with bacteria ..... 30

2.2.3 Restimulation with *E. coli* mpk2 ........................... 31

2.2.4 Sample collection from stimulated BMDCs .......... 32
2.2.4.1 Protein sample collection for Proteome analysis ...........................................32
2.2.4.2 Protein sample collection for Western blot.........................................................33
2.3 Detection of surface marker expression with FACS ........................................34
2.4 Measurement of cytokine secretion profiles from supernatants with ELISA ..........37
2.5 Western Blot for Prohibitin (PHB-1, anti-mouse) ................................................39
2.6 Measurement of reactive oxygen species (ROS) concentration .........................43
2.7 Proteomics run ................................................................................................45
2.8 Bioinformatics analysis and data analysis ..................................................45

3 Results ........................................................................................................46

3.1 *B. vulgatus* mpk and *E. coli* mpk2 induce different TNFα, IL-6, IL-12p40 and IL-1β secretion profiles ..................................................46
3.2 Stimulation with *B. vulgatus* mpk and *E. coli* mpk2 leads to differential expression of cell surface markers in BMDCs .........................49
3.3 Proteome analysis of stimulated DCs with commensal bacteria ........................51
3.3.1 Experimental setup ................................................................. 52
3.3.2 Determining differentially regulated proteins ........... 54
3.3.3 Clustering of the differentially regulated proteins .... 55
3.3.4 Detailed visualization of protein expression at multiple levels of detail ................................................. 57
3.4 Semi-mature DCs show higher expression of antioxidant and cytoprotective proteins, compared to fully mature dendritic cells .......... 73
3.5 Prohibitin (Phb1) amount is slightly increased in B. vulgatus mpk stimulated samples, compared to E. coli mpk2 stimulated samples .................... 78
3.6 DCs stimulated with pathobiotic bacteria harbor increased amounts of ROS, compared to DCs stimulated with symbiotic, protective bacteria ................................................................. 80
3.7 E. coli stimulation of BMDCs leads to higher surface expression of the migration marker CCR7 ........................................................................ 83

4 Discussion .................................................................................. 87
5 Conclusion .................................................................................. 113
6 Zusammenfassung ....................................................................... 116
7 Abbreviations ............................................................................. 120
Table of Figures

**Figure 1:** Illustrating phenotypical differences between DCs maturation states. ........................................8
**Figure 2:** Regulatory T-cells induction by semi-mature DCs .................................................................13
**Figure 3:** Culturing BMDCs ......................................................30
**Figure 4:** Stimulation of BMDCs ........................................31
**Figure 5:** Restimulation of BMDCs .................................32
**Figure 6:** Cytokine secretion profiles of cultured murine bone marrow derived dendritic cells, isolated from wild type C57BL/6. .......47
**Figure 7:** Expression of surface molecules by DCs upon stimulation with commensal bacteria...51
**Figure 8:** Distribution of proteins, annotated to major cellular processes, illustrating the
clustering of the proteome data with
DAVID annotation software..........................56

Figure 9: Differential protein expression of distinct
DC maturation states, visualized by
Voronoi treemaps........................................68

Figure 10: Expression ratios of proteins, involved
in stress response, in comparison between
semi-mature and fully mature DCs..............76

Figure 11: Prohibitin expression of semi-mature
and fully mature DCs.................................79

Figure 12: Production of reactive oxygen species
(ROS) upon stimulation with commensal
bacteria....................................................81

Figure 13: Expression of CCR7 by DCs upon
stimulation with commensal bacteria. ........84

Figure 14: Expression ratio of proteins, involved in
migration, in comparison between
semi- mature and fully mature DCs.............86

Figure 15: Akt1 signaling pathway....................94

Figure 16: Regulation of Keap1-Nrf2...............110
Table 1: Antibodies, used for FACS analysis. ............35
Table 2: Staining scheme with necessary controls. ....36
Table 3: Materials and buffers...............................38
Table 4: SDS-gel preparation ..................................40
Table 5: Buffers used for Western blot ......................42
Table 6: Stimulation scheme for ROS measurement ....44
Table 7: Description of different cases and statistical
tests used in data analysis.................................54
1 Introduction

1.1 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD), which include Crohn’s disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract. They are characterized by alternating phases of relapse and remission. Crohn’s disease patients present with fatigue, prolonged diarrhea (with or without bleeding), abdominal pain, fever and weight loss. CD may affect any part of the gastrointestinal tract and is pathologically defined as focal, transmural inflammation that may include stricturing (gut luminal narrowing), penetration (bowel perforation, fistula and abscess) and perianal disease (Lichtenstein, Hanauer, Sandborn, & Practice Parameters Committee of American College of, 2009; Satsangi, Silverberg, Vermeire, & Colombel, 2006; Wallace, Zheng, Kanazawa, & Shih, 2014). In comparison, UC predominantly involves the colon and the rectum and it is characterized by diffuse, continuous and symmetric inflammation, restricted to the colonic mucosa. Patients often present with rectal bleeding, frequent stools,
mucus discharge, tenesmus and lower abdominal pain (Kornbluth & Sachar, 1997; Satsangi et al., 2006; Wallace et al., 2014). Additionally, patients with IBD have increased risk for developing intestinal dysplasia, adenocarcinoma of the colon and extraintestinal disorders, such as psoriasis or primary sclerosing cholangitis (PSC) (Bernstein, Wajda, & Blanchard, 2005; Huang, Chandra, & Shih, 2012). About 0.3% of the European population (2.5-3 million people) suffers from IBD and there is a constantly increasing prevalence and incidence worldwide (Burisch, Jess, Martinato, Lakatos, & EpiCom, 2013). The highest incidence rates are observed in Scandinavia, whereas the lowest rates are in Southern, Eastern Europe, suggesting a north-west/south-east gradient. UC is more common than CD, however both diseases cause a high degree of distress for the patients, as a result of early onset, fluctuating course, serious complications, reduced quality of life and lack of cure. The etiology of IBD is still not clear. It is thought to be combination of genetics, microbiome, immune system and environment, which lead to abnormal immune response against the microflora in genetically susceptible individuals (Wallace et al., 2014).
Epidemiological data reports that many environmental factors, such as antibiotic use, microbial exposure (both early and late in life) and possibly diet, contribute to the development of the disease (Bager, Simonsen, Nielsen, & Frisch, 2012; De Filippo et al., 2010; Hviid, Svanstrom, & Frisch, 2011). Moreover, there are complex genetic factors behind IBD, which indicate the dysregulation of genes, associated with innate and adaptive immunity e.g. IL-23/Th17 pathway, TGF-β-pathway, TNF-pathway, NF-κB-activation and T-cell activation (Ek, D'Amato, & Halfvarson, 2014; Wallace et al., 2014). These genes resemble a small part of a bigger picture, if we are to understand the polymorphism of IBD. In our study, we investigated the role of the microbiome and its interaction with the innate immune system, hoping to contribute to the understanding of the pathogenic mechanisms underlying IBD.

1.2 Microbiota, *E. coli mpk2* and *B. vulgatus mpk*

The intestinal microbiota is a complex ecosystem, which resides on the 300-400m² gut luminal surface, in close contact with mucosal epithelial cells and the immune system (Wallace et al., 2014). It is comprised of bacteria,
archaea, protozoa, anaerobic fungi, and different bacteriophages and viruses, with an estimated 1000+ species of microbes in total (Turnbaugh et al., 2007). Genomics experiments have shown that 98% of all detected genes belong to bacteria and the three most abundant genera were Bacteroides, Prevotella and Ruminococcus, (Arumugam et al., 2011). The microbiome composition between individuals is variable and may change over time due to extrinsic factors, e.g. exposure to specific types of food and medications (e.g. antibiotics) or intrinsic factors, such as the adaptive immune system (Caporaso et al., 2011). Other genera, which have consistently been detected, include Actinobacteria, Proteobacteria, Fusobacteria, Faecalibacteria and Bifidobacteria. The intestinal microbiota has a considerable effect on the health and well-being of the human organism. It has metabolic functions (e.g. fermentation and absorption of carbohydrates), trophic effects on gut epithelial cells and plays a pivotal role in the suppression of pathologic microbial growth as well as in the development and improvement of the immune system (Dore & Corthier, 2010).
Individuals with IBD have been shown to have changes in the composition of their microbiome, compared to healthy individuals (Frank et al., 2007; Ott et al., 2004). Many studies report a decrease in members of the genera *Bacteroides* and increase of *Escherichia coli* (*E. coli*) in patients with ulcerative colitis (Chassaing & Darfeuille-Michaud, 2011; Lepage et al., 2011; Sokol, Lepage, Seksik, Dore, & Marteau, 2006; Wallace et al., 2014). Furthermore, in previous research, the commensal bacteria *Bacteroides vulgatus* mpk and *E. coli* mpk2 were investigated as to their capability to induce colitis in gnotobiotic IL-2−/− mice, which are a well-established model for microflora-triggered, chronic colitis in hosts (Autenrieth, Bucheler, Bohn, Heinze, & Horak, 1997; Sadlack et al., 1993). Germ-free and *B. vulgatus mpk* mono-colonized mice remain healthy, whereas *E. coli* mpk2 mono-colonized animals developed the disease. Moreover, a co-colonization with *E. coli* mpk2 and *B. vulgatus* mpk did not lead to colitis, suggesting, that *B. vulgatus* mpk has protective properties, which remain to be further elucidated.
In this study we investigate the maturation process of dendritic cells upon stimulation with colitogenic and non-colitogenic commensal bacteria and their effect on intestinal inflammation and IBD. For this purpose, we used the commensal *E. coli* mpk2 strain, which induces colitis, and the commensal *B. vulgatus* mpk strain, which prevents *E. coli* mpk2 induced colitis in IL-2−/− mice.

### 1.3 Dendritic cells and their role in IBD.

As the gastrointestinal tract represents one of the main entrances for pathogenic invasion, it has to be tightly controlled by the host immune system. Therefore, about 80% of all leukocytes in human body reside in the gut associated lymphatic tissue (GALT), interacting with each other in a very complex manner, which is still not fully understood. An effective immune response to infectious agents or inflammatory products demands perfectly coordinated teamwork between the innate and adaptive immune system. Dendritic cells (DCs) are antigen presenting cells (APCs), which bridge the gap between innate and adaptive immunity. In comparison to other mononuclear phagocytes (MPs) such as monocytes and macrophages, DCs are distinguishable by their unique
morphology and ability to capture and process antigens, which are subsequently presented to naïve T-cells (Rutella & Locatelli, 2011). Dendritic cells, located in the intestines, are in close proximity to the luminal surface (Rescigno et al., 2001) and exert a strict control over the antigen traffic in the gut. They play a dual role in inducing an adaptive immune response against potentially harmful antigens, on one hand, and maintaining T-cell tolerance to self-antigens and commensal microbiota on the other (Steinman & Banchereau, 2007). Upon confrontation with pathogens and activation, DCs undergo maturation, which can be seen as a preparation for an effective communication with naïve T-cells. The maturation process is characterized by upregulation of major histocompatibility complex class II (MHC-II) and costimulatory molecules (e.g. CD40), as well as cytokine production and the ability to migrate and activate T-cells (Figure 1). Based on specific phenotypical appearance, as defined by the aforementioned features, three distinct maturation states were described: immature, semi-mature and fully mature DCs (Lutz & Schuler, 2002).
Introduction

Figure 1: Illustrating phenotypical differences between DCs maturation states.
The image is adopted and modified from (Lutz & Schuler, 2002). Immature DCs (green) are characterized by low expression of surface markers (MHC-II and CD40) and no pro-inflammatory cytokine secretion. They are able to induce T-cell anergy. Semi-mature DCs (blue) express more MHC-II and CD40, however lack of cytokine secretion. They are irresponsible to a second maturation stimulus and are proposed to induce Treg cells. Fully mature DCs (red) are distinguishable by the high amount of pro-inflammatory cytokines produced and their ability to induce T-cell mediated inflammation.

Immature dendritic cells are resting APCs in peripheral tissues, such as the epidermis (Langerhans cells) or the lung epithelium, or the gastrointestinal mucosa. Functionally, they are characterized by continuous endocytosis, which does not lead to activation, maturation
or increased migration to lymph nodes. If stimulation does not take place they remain in a steady state in which they are sensing the environment, expressing small quantities of MHC-II and a basal level of costimulatory molecules (Lutz & Schuler, 2002; Mommaas et al., 1995). Without costimulatory molecules immature DCs cannot activate T-cells, which leads to clonal T-cell anergy (Lutz & Schuler, 2002; Schwartz, Mueller, Jenkins, & Quill, 1989). Immature DCs normally do not migrate, however spontaneous migration in the absence of stimulatory factors can occasionally occur (Lutz & Schuler, 2002; Randolph, 2001). In our experiment immature DCs are represented by unstimulated bone marrow-derived dendritic cells (BMDCs).

The semi-mature state of DCs is defined as partial maturation with upregulation of both MHC-II and costimulatory molecules (e.g. CD40), but a lack of proinflammatory cytokine production such as TNFα, IL-1β, IL-6 and IL-12b. In contrast with immature DCs, semi-mature DCs demonstrate improved migration properties, which enables them to reach T-cells at their anatomical location (Lutz, 2012; Lutz & Schuler, 2002). The
induction of partially maturated DCs can be achieved in diverse ways (e.g. TNF- stimulation, LPS stimulation, bacterial stimulation) (Lutz & Schuler, 2002). In our experiment, we generate semi-mature DCs via stimulation with the symbiotic, non-colitogenic B. vulgatus mpk, which has been previously established by (Frick et al., 2006) differing from the TNF induced semi-mature DCs, proposed by Lutz et al. (2002). The B. vulgatus mpk induced semi-mature DCs express intermediate levels of MHC-II and CD40 and low levels of proinflammatory cytokines. Furthermore, the semi-mature state was characterized by unresponsiveness to a second maturation stimulus (Frick et al., 2006; Perrot et al., 2007), indicating that semi-maturation is rather a distinct state and not an intermediary phase on the way to full maturation. Although the use of the term “semi-maturation” stays under debate, there is a high number of studies which have provided evidence for the existence of these cells and their tolerogenic potential (Braun et al., 2006; Frick, Grunebach, & Autenrieth, 2010; Lutz, 2012; Mills & McGuirk, 2004; Morel & Turner, 2011; Morelli et al., 2005; Nouri-Shirazi & Thomson, 2006; Rutella, Danese,
& Leone, 2006; van Duivenvoorde, van Mierlo, Boonman, & Toes, 2006; Young, Merad, & Hart, 2007). Our proteomics experiment provides a detailed catalogue of differentially expressed proteins, aiming to characterize and distinguish the semi-mature state from the other two well-known maturation states.

The potential of semi-mature DCs to induce peripheral tolerance depends on their ability to activate different types of regulatory T-cells (Belkaid & Oldenhove, 2008; Lutz, 2012). Regarding their origin, we differentiate between thymically-derived (natural) Treg cells and inducible Treg cells in the periphery. Regarding their phenotype, there are IL-10 producing T-regulatory cells (Tr1), TGFβ- producing T-helper cells (Th) and inducible Foxp3+ Treg (Belkaid & Oldenhove, 2008; Chen et al., 2003). The main function of Treg cells is to control peripheral homeostasis by limiting extensive immune response, initiated by effector T-cells. Under normal conditions, natural, thymically-derived Treg cells are sufficient to maintain peripheral homeostasis. However, the generation of additional, extrathymical Treg cells is required on certain occasions and in specific
environments, which are continuously exposed to microbes, e.g. the gastrointestinal tract. Under specific bacterial stimuli, dendritic cells develop the ability to induce new populations of antigen-specific Treg cells (Belkaid & Oldenhove, 2008) and thus play a key role in preserving intestinal integrity. Although the exact mechanism is incompletely understood, it has been proposed that DCs which secrete IL-10 lead to the development of IL-10⁺ Tr1 cells, whereas TGFβ producing DCs induce Foxp3⁺ Treg cells (Lutz, 2012) (Figure 2). Our results may provide new ideas on the induction of Treg cells by dendritic cells, interacting with commensal bacteria, since we detect potential regulators of peripheral tolerance in semi-mature DCs.
**Introduction**

![Figure 2: Regulatory T-cells induction by semi-mature DCs](image)

**Figure 2: Regulatory T-cells induction by semi-mature DCs**

The image is adopted and modified by Lutz et al. 2012. Stimulation of immature DCs (green) leads to the development of two different semi-mature DCs. The IL-10 producing one on the left side induces IL-10+Tr1 cells and the TGFβ producing DC on the right induces Foxp3+ Treg cells.

Fully mature DCs are mainly characterized by their potential to produce proinflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-12b. Furthermore, they express high levels of MHC-II and costimulatory molecules and are crucial for the induction of T-cell immunity (Lutz, 2012; Lutz & Schuler, 2002). Additionally, fully mature DCs are characterized by increased CCR7 expression and
the ability to migrate to secondary lymphatic organs such as regional lymph nodes or the spleen (Alvarez, Vollmann, & von Andrian, 2008; Johnson & Jackson, 2014; Saban, 2014; Teijeira, Russo, & Halin, 2014). Mechanistically, increased cytokine production is induced via signals, associated with pathogen recognition such as pathogen-associated molecular patterns (PAMPs) and their detection by pattern-recognition receptors (PPRs) (Lutz & Schuler, 2002). An example of such receptors are toll-like recognition receptors (TLRs), which were first discovered in *Drosophila* (Medzhitov, Preston-Hurlburt, & Janeway, 1997) and were later found on the surface of antigen presenting cells such as DCs. APCs use TLRs as sensors, which are crucial for the innate immune system to decode the type of invading pathogen and initiate an appropriate immune response. There are 13 different TLR proteins, each specific for different PAMPS. For example, TLR2 detects bacterial peptidoglycans, TLR3 – double stranded DNA, TLR4 – lipopolysaccharide (LPS), TLR5- bacterial flagella and TLR9 – viral DNA rich in unmethylated CpG motifs (Lu, Yeh, & Ohashi, 2008). One of the best studied interactions is the one between TLR4 and LPS, an
important structural component of the outer membrane of Gram-negative bacteria (in our study *B. vulgatus* mpk and *E. coli* mpk2). LPS stimulation involves many proteins, including LPS-binding protein (LBP), CD14, MD-2 and TLR4. CD14 facilitates the transfer of LPS to the TLR4/MD2 receptor complex and modulates LPS recognition (Lu et al., 2008). Subsequently, a series of intracellular cascades are induced, which can be divided into MyD88-dependent and MyD88-independent pathways. The first one leads to the production of multiple pro-inflammatory cytokines and the second one to Type 1 Interferon secretion (Lu et al., 2008).

Other mechanisms, involved in PAMPs recognition, include intracellular NOD-like receptors (NLRs) and activation of the inflammasome. Inflammasomes are the interface between TLRs and NLRs and resemble multiprotein complexes that are responsible for the activation of inflammatory caspases. Caspase 1, for example, is required for the cleaving of the biologically inactive IL-1β and thus controls its activation (Church, Cook, & McDermott, 2008). IL-1β is one of the most important cytokines during inflammation and is primarily
produced by cells of the monocytic linage (Dinarello, 1996; Eder, 2009). It causes fever, hypotension and production of additional proinflammatory cytokines (Church et al., 2008). The release of IL-1β encompasses three steps: (i) transcription of the biologically inactive pro-IL-1β, (ii) cleavage of pro-IL-1β by caspase-1 and (iii) secretion of the biologically active, mature IL-1β into the extracellular milieu (Eder, 2009). Overproduction of IL-1β can be detrimental for the surrounding tissue and has been reported to be associated with wide variety of diseases, including atherosclerosis, type 2 diabetes, neurodegenerative and autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis and Crohn’s disease (Church et al., 2008).

Although DC maturation is an extensively investigated topic in the literature, the underlying molecular patterns creating the difference between immature, semi-mature and fully mature DCs, are only marginally understood. The purpose of our experiment is to provide a more detailed picture of the maturation process of DCs, using proteomics as a tool.
1.4 Reactive oxygen species

ROS are highly reactive, oxygen containing molecules, which include free radicals i.e. superoxide anion (\(\cdot \text{O}_2^\cdot\)), hydroxyl radical (\(\cdot \text{OH}\)), peroxyl radical (\(\text{ROO}\cdot\)), alkoxyl radical (\(\text{RO}\cdot\)) and thiol radical (\(\text{RS}\cdot\)), as well as non-radicals such as hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and singlet oxygen (\(^1\text{O}_2\)) (Oyinloye, Adenowo, & Kappo, 2015).

ROS can originate from external and internal sources. We are constantly exposed to external sources, e.g. ionizing radiation, ultra violet (UV) light and general pollution, such as smoking, car exhaust fumes and factory fumes. Internal sources include, on the one hand, innate immune cells involved in killing invading pathogens in a process called “oxidative burst” and; on the other hand, the electron transport chain (ETC) in the mitochondria, which is crucial for the metabolism of all body cells. It is believed that around 1% of all passing electrons are leaking from the ETC and thereby reducing \(\text{O}_2\) to superoxide radicals, which are subsequently converted to hydrogen peroxide by the superoxide dismutase (SOD). In general, ROS are harmless at low concentrations and play an important role
as second messengers in many physiological processes and cell signaling cascades. However, under certain circumstances, such as pathogen confrontation, inflammation and injury, ROS production may rise to levels that overwhelm the antioxidant capacity of the cell. Prolonged exposure to oxidative stress causes damage to important structural components i.e. lipids, proteins, carbohydrates and deoxyribonucleic acid (DNA) (Alfadda & Sallam, 2012; Martinon, 2010; Pham-Huy, He, & Pham-Huy, 2008). For example, by reacting with polyunsaturated fatty acids of cell membranes, free radicals are able to initiate a vicious chain reaction, referred to as lipid peroxidation, which subsequently leads to membrane destruction (Del Rio, Stewart, & Pellegrini, 2005; Pham-Huy et al., 2008). Proteins can also be damaged by the oxidation of amino acids, especially cysteine residues, leading to structural changes and enzymatic dysfunction (Halliwell, 2007). Furthermore, damage to DNA can be caused by ROS, inducing single oxidative lesions or strand brakes (Meira et al., 2008). If not properly repaired by DNA repair mechanisms, these changes can contribute to the development of deleterious
mutations and cancer. Oxidative stress has been linked to the pathogenesis of many chronic and degenerative diseases such as autoimmune disorders, arthritis, cancer, cardiovascular and neurodegenerative disease (Martinon, 2010). There is a growing body of evidence, which show the important role of ROS in inflammatory bowel disease, however the exact mechanism and extent of involvement needs to be further studied.

1.5 ROS and “leaky gut” syndrome

Recently, ROS was associated with disruption of the intestinal epithelial barrier (Banan, Choudhary, Zhang, Fields, & Keshavarzian, 2000; A. Wang et al., 2014), which is characterized by increased epithelial permeability. As a result of altered intestinal permeability, bacteria and food antigens may enter the submucosa and induce severe inflammation. By invading the gut tissue, pathogens and inflammatory products gain access to the circulatory and lymphatic system, which can lead to disseminated spread to other organs, sepsis and death (A. Wang et al., 2014). This pathogenesis has been referred to as “leaky gut syndrome” and has been proposed as an additional hypothesis for explanation of autoimmune
diseases (Fasano, 2012). There is an increasing body of evidence demonstrating an association of gut epithelial barrier dysfunction with other somatic disorders, e.g. heart disorders, diabetes mellitus type I, cancer, IBD, asthma, multiple sclerosis, ankylosing spondylitis (Fasano, 2012; Rogler & Rosano, 2014; Saggioro, 2014), and mental disorders e.g. autism (Samsam, Ahangari, & Naser, 2014). We believe that the disruption of the intestinal mucosal barrier by ROS contributes to disease pathology in autoimmune diseases, especially in IBD.

1.6 Antioxidants, Nrf2-pathway and Prohibitin (Phb1).

To prevent excessive ROS production and tissue damage, living cells are equipped with defense mechanisms, classified as enzymatic antioxidants and non-enzymatic antioxidants. The main enzymes that deal with radicals are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx). SOD facilitates as a first line of defense in the mitochondria- SOD (Mn), or in the cytoplasm- SOD (Cu/Zn), the dismutation of two superoxide anions to oxygen and hydrogen peroxide. The H₂O₂ is neutralized to
Introduction

oxygen and water either by catalase (CAT), or glutathione peroxidase (GPx). The latter removes hydrogen peroxide by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG), which is then reduced back to GSH by the flavoprotein enzyme Glutathione reductase (Pham-Huy et al., 2008).

Non-enzymatic antioxidants are divided into metabolic antioxidants, produced in the body, and nutrient antioxidants, absorbed from food and food supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids (Pham-Huy et al., 2008). The most important metabolic antioxidants include glutathione, L-ariginine, coenzyme Q10, melatonin and metal-chelating proteins. In general, when an antioxidant destroys a free radical, this antioxidant itself becomes oxidized and must be either detoxified by another antioxidant, or it remains unreactive by delocalizing of its free electrons. The latter is characteristical for antioxidants, rich in conjugated double bounds, such as Vit. C, E, Coenzym Q10 etc.

A key regulator in the endogenous antioxidant system is the nuclear factor erythroid 2-related factor 2
(Nrf2). This is a transcription factor, responsible for the activation of a great variety of cytoprotective genes, including antioxidant, detoxification and metabolic control enzymes (Hybertson & Gao, 2014). Under steady state, Nrf2 is repressed by the redox sensitive kelch-like ECH-associated protein 1 (Keap1). Upon reaction with ROS or other electrophiles, Keap1 releases Nrf2 allowing it to enter the nucleus, where it initiates the transcription of antioxidant response elements (AREs). Among other major representatives of AREs there are important antioxidant enzymes i.e. superoxide dismutase (Sod1 Cu/Zn), glutathione s-transferase omega-1 (Gsto1), thioredoxin (Txn) and glutamate-cysteine ligase (Gcl) (Hayes & Dinkova-Kostova, 2014; Higgins et al., 2009; Theiss, Vijay-Kumar, et al., 2009). The aforementioned SOD is one of the integral enzymes for detoxifying peroxide anions to hydrogen peroxide in the mitochondria and cytoplasm of the cell. Gsto1 and Gclm are crucial proteins for intracellular glutathione homeostasis (McMahon et al., 2001). As mentioned before, glutathione is responsible for neutralizing the product of SOD - $\text{H}_2\text{O}_2$ in the cell plasma and thus to completely restore the redox
balance in the cell (McMahon et al., 2001). Besides the GSH based antioxidant system, Nrf2 controls the expression of cytoplasmic thioredoxin, which is responsible for the reduction of oxidized protein thiols, common products of glutathione oxidation (Hawkes, Karlenius, & Tonissen, 2014; Hayes & Dinkova-Kostova, 2014). Thus, Txn, together with Gsto1, Sod and Gclm represents a major member of the antioxidant capacity of the cell.

The activation of Nrf2/Keap1 pathway is tightly controlled. Under normal homeostatic conditions, Nrf2 is kept at low levels by targeted proteosomal degradation by ubiquitination (Hayes & Dinkova-Kostova, 2014). The expression of the transcription factor is regulated on a genetic (gene polymorphism), transcriptional, translational and post-translational levels (Hayes & Dinkova-Kostova, 2014). Furthermore, there are regulator proteins such as Prohibitin (Phb1) and p62/sequestosome 1 (p62/SQSTM1), which can influence the Nrf2/Keap1 pathway by different mechanisms. P62/SQSTM1, for example, inhibits Keap1 by competing with Nrf2 for the binding site and thus positively regulating Nrf2 expression.
Introduction

(Copple et al., 2010; Hayes & Dinkova-Kostova, 2014; Komatsu et al., 2010). Prohibitin (Phb1) has also been shown to exert a positive regulation on the major antioxidant pathway, however the mechanism is not completely understood (Theiss, Vijay-Kumar, et al., 2009). Phb1 is an ubiquitously expressed, highly conserved protein, which has multiple functions in the cell including regulation of proliferation and apoptosis, regulation of transcription, and mitochondrial protein folding (Theiss & Sitaraman, 2011). Phb1 was demonstrated to be downregulated during oxidative stress in cultured intestinal epithelial cells, IBD and mice models of colitis (Theiss et al., 2007; Theiss, Vijay-Kumar, et al., 2009). The same research group demonstrated a clear relationship between Prohibitin overexpression in the intestines and upregulation of glutathione during oxidative stress. Phb repletion was further shown to protect from oxidant-induced epithelial barrier dysfunction and colitis in mice models (Theiss, Vijay-Kumar, et al., 2009). Due to its beneficial regulatory properties, Prohibitin is now the target of new therapeutic approaches in IBD (Theiss & Sitaraman, 2011).
1.7 Aims

Dendritic cell maturation is an extensively investigated topic, however, the underlying molecular patterns creating the differences between immature, semi-mature and fully mature DCs are marginally understood. The aim of this doctoral thesis is to investigate the interaction of symbiotic and pathobiotic bacteria with dendritic cells and gaining more insight into the pathogenic mechanisms underlying IBD. By using proteomics as a tool, we intend to provide a more detailed picture of the maturation process of DCs by generating a comprehensive list of differentially regulated proteins between distinct DC maturation states. Moreover, we intend to provide functional clues by systematically clustering these proteins into biological processes and effectively visualizing them on Voronoi treemaps in multiple levels of detail. Relevant processes e.g. inflammation, migration and stress response will be further investigated to reveal regulatory proteins that may play a crucial role in creating the differences between semi-mature and fully mature DCs. Finally, we will explore in detail ROS generation in dendritic cells upon
bacterial challenge, in conjunction with the different antioxidant capacities of distinct DC maturation states, as demonstrated in our proteomics experiment.
Materials and Methods

2 Materials and Methods

2.1 Bacterial cultures

2.1.1 *Escherichia coli* mpk2

5ml of LB (Lysogeny Broth)-medium was inoculated with 50µl *E. coli* mpk2 stock solution and incubated overnight on a shaker at 37°C. The next day, 300µl of the overnight culture was used to inoculate 20ml of fresh LB-medium for 2.5 hours at 37°C on a shaker.

2.1.2 *Bacteroides vulgatus* mpk

Four days before usage, 50µl from *B. vulgatus* mpk stock solution was used to inoculate 10ml liver-bouillon and was incubated under anaerobic conditions, at 37°C for 3 days. After incubation, 5ml of the three-days-old culture was used to inoculate 45ml BHI (Brain Heart Infusion)-medium and the suspension was kept overnight under anaerobic conditions at 37°C.
2.2 Cell culture

2.2.1 Bone marrow derived murine dendritic cells (BMDCs)

2.2.1.1 Cell culture medium for BMDCs

The medium used for generating BMDCs out of primary bone marrow cells consists of 500ml VLE-RPMI-medium (Biochrom), supplemented with 10% heat inactivated (57°, 30min) fetal calf serum (FCS, Sigma), 1% non-essential amino acids (Biochrom), 100 Units/ml Penicillin (Invitrogen), 100µg/ml Streptomycin (Invitrogen), 50µmol/l 2-Mercaptoethanol (Sigma) and 10% GM-CSF (Granulocyte macrophage colony-stimulating factor), produced by the murine myeloma cell line P3X63 in house.

2.2.1.2 Isolation and culturing of BMDCs

Primary bone marrow cells were isolated from the femur and tibia of C57BL/6 mice and cultured according to a protocol established previously (Madaan, Verma, Singh, Jain, & Jaggi, 2014). Mice were killed by CO₂ asphyxiation, followed by cervical dislocation. Femur and tibia were separated from the surrounded tissue.
with sterile instruments. After removal, the bones were plated in sterile Phosphate Buffered Saline (PBS [+] MgCl₂ [+] CaCl₂ [+], GIBCO). In a class two biosafety cabinet, the bones were cleaned from muscular tissue and separated from the knee joint. Epiphysis were cut and the bone marrow was flushed with PBS out of the diaphysis on a cell filter (BD Falcon, 100μm), using a syringe (Braun). Cell suspension was subsequently spun down for 5min at 400g, supernatant was taken out and cells were resuspended in 10ml BMDC medium. After counting with a hemocytometer, 2 x 10⁶ cells were cultured in 10ml BMDC-medium in a 10cm petri dish (Falcon) and kept in a cell culture incubator. Medium was refreshed on days 3 and 6 after bone marrow isolation. Generated dendritic cells are harvested after seven days of culture for experiments. All animal experimentations are done by qualified personal and confirm to the rules and guidelines of University Hospital Tübingen and the land of Baden-Württemberg.
2.2.2 In vitro dendritic cell stimulation with bacteria

On day 7 of DC culture, the cells were scraped out and replated on 6-12-well plates (Falcon, non-tissue treated) for stimulation, using 1ml medium per $1 \times 10^6$ cells. After 2 hours in the cell incubator, DCs were inoculated with *B. vulgatus* mpk and *E. coli* mpk2 with a MOI (Multiplicity of infection) of 1 and incubated further for 5 min and 16 hours in the cell culture incubator. 1µg/ml of Gentamycin was added to the culture to prevent multiplication of the bacteria (Figure 4).
Figure 4: Stimulation of BMDCs
Stimulation of cultured immature dendritic cells with *B. vulgatus* mpk (MOI 1) and *E. coli* mpk2 (MOI 1). Samples were collected 5 minutes and 16 hours after stimulation and prepared for proteome analysis.

2.2.3 Restimulation with *E. coli* mpk2

After refreshing the medium, cells previously stimulated with *B. vulgatus* mpk and *E. coli* mpk2 as well as the unstimulated control cells, were restimulated with *E. coli* mpk2 (MOI 1) for another 16 hours (Figure 5).
Materials and Methods

Figure 5: Restimulation of BMDCs
_E. coli_ mpk2 restimulation of untreated, _B. vulgatus_ mpk and _E. coli_ mpk2 pretreated cultures. After 16h samples were collected and analyzed for maturation check with FACS and ELISA.

2.2.4 Sample collection from stimulated BMDCs

2.2.4.1 Protein sample collection for Proteome analysis

Protein samples were collected 5 minutes and 16 hours after stimulation for each of the three conditions: treated with _E. coli_ mpk2, treated with _B. vulgatus_ mpk and untreated. After incubation with bacteria, cells were scraped and washed with sterile PBS. After centrifugation, the pellet was resuspended in 0.5ml of lysis solution (6M urea, 2M thiourea in 50mM Tris pH 8.0, 1% N-Octylglucoside + phosphatase inhibitors: 1mM Sodium
orthovanadate, 5mM Glycerol-2-phosphate, 5mM Sodium fluoride). After ten minutes incubation on ice, 0.5% benzonase was added to the suspension and kept for 12min at room temperature, in order to digest DNA. After incubation, samples were centrifuged at 2800g, 10°C for 22 minutes and the supernatant was frozen for subsequent proteome analysis.

2.2.4.2 Protein sample collection for Western blot

The protein samples for Western blot were collected similarly to those for proteome analysis: 5 minutes and 16 hours after stimulation for all the three conditions. Cells were scraped and washed twice with PBS. Afterwards the cell pallet was resuspended in 100µl of lysis buffer (1% Triton-X-100, diluted in PBS + Protease inhibitor (1 Tablet in 2ml distillated water and mix 1:25 with the 1% Triton-X-100)). After rotation at 4°C for 15min, cells were centrifuged at 8000rpm 4°C and stored at -20°C.
2.3 Detection of surface marker expression with FACS

After 5 min and 16 hours of incubation, the cells were scraped out and centrifuged. Pellets were resuspended in 150µl Fc-Block and incubated (+4°C, 15min) in darkness. After washing with PBS + 1% FCS, 150µl cell suspension was stained with 0.7µl monoclonal antibodies according to Table 2 below. Cells were incubated with the antibodies (+4°C, 45min) in darkness and washed again with PBS + 1% FCS. Eventually, the pellet was resuspended in 150µl PBS + 1% FCS and transferred to FACS tubes (Greiner). 50µl of 4% PFA (Paraformaldehyde) was added for preservation. The samples were analyzed using BD LSRSortessa II cell analyzer (BD) for detecting the expression of cell surface activation markers CD40, MHC-II and CCR7 as well as the dendritic cell marker CD11c (see Table 1). The results from the flow cytometer were further analyzed using the Flow Jo software.
Table 1: Antibodies, used for FACS analysis.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Clone</th>
<th>Isotype</th>
<th>Catalog No./ Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD 40 FITC</td>
<td>HM40-3</td>
<td>Hamster IgM, κ</td>
<td>553723, BD</td>
</tr>
<tr>
<td>MHC-II FITC</td>
<td>2G9</td>
<td>Rat IgG2b, κ</td>
<td>553623, BD</td>
</tr>
<tr>
<td>CD11c APC</td>
<td>HL3</td>
<td>Hamster IgG1, λ2</td>
<td>550261, BD</td>
</tr>
<tr>
<td>CD11c FITC</td>
<td>HL3</td>
<td>Hamster IgG1, λ2</td>
<td>557400, BD</td>
</tr>
<tr>
<td>CCR7 Biotin</td>
<td>4B12</td>
<td>Rat IgG2a, κ</td>
<td>13-1971-82, eBio</td>
</tr>
<tr>
<td>Streptavidin APC</td>
<td></td>
<td></td>
<td>554067, BD</td>
</tr>
</tbody>
</table>
### Table 2: Staining scheme with necessary controls.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cells used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Unstained</td>
<td>Cell mixture</td>
</tr>
<tr>
<td>2. MHC-II FITC single staining</td>
<td></td>
</tr>
<tr>
<td>3. CD40 FITC single staining</td>
<td></td>
</tr>
<tr>
<td>4. CD11c APC single staining</td>
<td></td>
</tr>
<tr>
<td>5. CCR7 Bio + Strep. APC single staining</td>
<td></td>
</tr>
<tr>
<td>6. CD11c APC, MHC-II FITC</td>
<td>Non-stimulated cells</td>
</tr>
<tr>
<td>7. CD11c APC, CD40 FITC</td>
<td></td>
</tr>
<tr>
<td>8. CD11c FITC, CCR7 Bio + Strep.APC</td>
<td></td>
</tr>
<tr>
<td>9. CD11c APC, MHC-II FITC</td>
<td>B. vulgatus</td>
</tr>
<tr>
<td>10. CD11c APC, CD40 FITC</td>
<td>stimulated cells</td>
</tr>
<tr>
<td>11. CD11c FITC, CCR7 Bio + Strep.APC</td>
<td></td>
</tr>
<tr>
<td>12. CD11c APC, MHC-II FITC</td>
<td>E. coli stimulated cells</td>
</tr>
<tr>
<td>13. CD11c APC, CD40 FITC</td>
<td></td>
</tr>
<tr>
<td>14. CD11c FITC, CCR7 Bio + Strep.APC</td>
<td></td>
</tr>
</tbody>
</table>
2.4 Measurement of cytokine secretion profiles from supernatants with ELISA

At 16 hours after stimulation with *B. vulgatus* mpk, *E. coli* mpk2 and PBS for control, as well as the restimulated samples were centrifuged and supernatants were frozen for cytokines detection with ELISA. The following BD OptEIA-sets were used: TNFα (anti-mouse, catalog No: 554418, BD), IL-1β (anti-mouse, catalog No: 559603) and IL-6 (anti-mouse, catalog No: 554401, BD). Maxi Sorb 96-well plates (Nunc) were coated overnight (4°C) with 100µl capture antibody (purified anti-mouse mAb). Afterwards, the plates were washed (2x) with ELISA-wash buffer and blocked, using 200µl Assay Diluent for 1 hour. Standards from the respective OptEIA-sets were diluted accordingly, to obtain a standard curve and together with the probes, they were incubated in the already washed (1x) plate for 2 hours at room temperature. After 2 washing steps 100µl detection antibody (biotinylated anti-mouse mAb) + HRP (horse radish peroxidase)-streptavidine-enzyme, diluted 1:250 in Assay Diluent was added and the plate was incubated further for 1 hour at room temperature. 100µl substrate solution was
added per well to the pre-washed plate (2x) and after 20min incubation in darkness the reaction was stopped with 50µl sulfuric acid. The optical density (OD) of the probes was measured using the ELISA-reader and Magellan-V 6.6 as a software.

**Table 3: Materials and buffers**

<table>
<thead>
<tr>
<th>Material/Buffer</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maxi Sorb 96-well plates</td>
<td>Nunc</td>
</tr>
<tr>
<td>ELISA-Washer</td>
<td>Tecan</td>
</tr>
<tr>
<td>ELISA- wash buffer</td>
<td>1 x PBS + 0.05 % Tween-20</td>
</tr>
<tr>
<td>Coating buffer</td>
<td>0.2 mol/l sodium phosphate - pH 6.5</td>
</tr>
<tr>
<td></td>
<td>IL-6 - 0.1 mol/l</td>
</tr>
<tr>
<td></td>
<td>sodium carbonate - pH 9.5</td>
</tr>
<tr>
<td>Assay Diluent</td>
<td>1 x PBS + 10 % FCS</td>
</tr>
<tr>
<td>Substrate solution</td>
<td>tetramethylbenzidine + hydrogen peroxide 1:1 (TMD-reagents-set, BD)</td>
</tr>
<tr>
<td>Stop-solution</td>
<td>50µl sulfuric acid 1 mol/l</td>
</tr>
<tr>
<td>ELISA-reader</td>
<td>Tecan SunRise</td>
</tr>
</tbody>
</table>
2.5 Western Blot for Prohibitin (PHB-1, anti-mouse)

Protein collection for Western blot was described above (section 1.4.2). Protein concentration in samples was measured with Bradford-assay, which is based on the reaction of the Triphenylmethan Coomasie-Brilliant-Blau G-250 in an acidic milieu with the cationic, nonpolar side chains of a protein. Protein concentration was measured according to the photometrical results at the absorption maximum (595nm). BSA protein standards are used to obtain a standard curve. 30µg proteins per sample was loaded in a SDS-gel (see Table 4), dissolved in 15µl solution, consisting of 5x Laemmli-puffer, as a loading dye, and the rest – millipore water. 3µl molecular marker (PAGE Ruler Protein Ladder) was used as size standard to monitor the progress of the electrophoresis. The SDS-Page ran under 30mA and constant voltage for 2 hours.
Table 4: SDS-gel preparation

<table>
<thead>
<tr>
<th></th>
<th>Millipore water</th>
<th>30% acrylamide mix</th>
<th>1.5M Tris (pH 8.8)</th>
<th>10% SDS</th>
<th>10% APS</th>
<th>TEMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% separating gel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stacking gel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After the SDS-Page run, a blot-sandwich in a wet blot puffer was prepared for transferring the proteins into a nitrocellulose membrane. The blotting ran at 300mA for 1 hour. After run, the nitrocellulose membrane was incubated in LiCor Blocking Buffer (1h, at room temperature), than washed (3x10min) with Western Wash and eventually incubated with the primary antibody Prohibitin (Rabbit anti-PHB-1, cell signaling #2426), diluted in 5ml LiCor Blocking Buffer and 0.1% Tween 20 and kept overnight, at 4°C, under rotation. On the next day,
the membrane was washed (3x10min) and incubated with the secondary antibody (goat, anti-rabbit IRDye 680RD), diluted in 5ml LiCor Blocking Buffer, 0.2% Tween 20 and 0.1% SDS and kept for 2h at room temperature, under rotation. The 700nm and 800nm fluorescence signal was measured with Li-Cor Odyssey. After obtaining results from the first staining, the membrane was stripped using strongly alkali stripping solution (10mM NaOH, 250 mM Guanidiniumchlorid) and stained again with β-Actin antibody (mouse, anti-β-Actin, catalog no. 4970, Cell signaling Technology) for loading control. The membrane was incubated for 1h with the primary antibody than washed (3x10min) and incubated for 45min with the secondary antibody (goat, anti-mouse IRDye 680RD). Fluorescence was measured once again by Li-Cor Odyssey.
### Table 5: Buffers used for Western blot

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Laemmli-buffer</td>
<td>2.5ml Tris (1M, pH 6.8)</td>
</tr>
<tr>
<td></td>
<td>5ml Glycerin</td>
</tr>
<tr>
<td></td>
<td>1g SDS</td>
</tr>
<tr>
<td></td>
<td>0.5% Bromophenol blue</td>
</tr>
<tr>
<td></td>
<td>Fill up to 10ml with aqua dest.</td>
</tr>
<tr>
<td>5x Electrophoresis buffer</td>
<td>50mM Tris</td>
</tr>
<tr>
<td></td>
<td>384mM Glycine</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
</tr>
<tr>
<td></td>
<td>Fill up to 1L with aqua dest.</td>
</tr>
<tr>
<td>10x Western blot buffer</td>
<td>25mM Tris, pH 8.3</td>
</tr>
<tr>
<td></td>
<td>192mM Glycine</td>
</tr>
<tr>
<td></td>
<td>0.02% SDS</td>
</tr>
<tr>
<td></td>
<td>20% Methanol</td>
</tr>
<tr>
<td>Western wash buffer</td>
<td>500mL PBS</td>
</tr>
<tr>
<td></td>
<td>0.1% Tween 20</td>
</tr>
</tbody>
</table>
Materials and Methods

2.6 Measurement of reactive oxygen species (ROS) concentration

Concentration of ROS was measured using the chemical 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), which reacts with cellular esterases and ROS to the fluorescent dichlorofluorescein (DCF), after diffusing into the cell. The fluorescence signal, which corresponds to ROS levels, was measured with FACS, using FITC channel at 480nm excitation and 530nm emission. 50mg of DCFH-DA powder was dissolved in 10ml Dimethylsulfoxid (DMSO) and aliquots were stored at -20°C in darkness. The concentration of the dye in the frozen stock solutions was 10mM. For positive controls a concentration of 500µM and 1000µM H₂O₂ was used. After harvesting and counting, 1x10⁶ immature dendritic cells per 1ml culture medium were put in a 12 well plate (non-tissue treated, FALCON), as shown in Table 6, and stored in a cell culture incubator.
Materials and Methods

Table 6: Stimulation scheme for ROS measurement

<table>
<thead>
<tr>
<th>1x10^6 cells</th>
<th>1x10^6 cells + B. vulgatus MOI 1</th>
<th>1x10^6 cells + E. coli MOI 1</th>
<th>1x10^6 cells + E. coli MOI 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>(non-stimulated)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10^6 cells + 500µM H_2O_2</td>
<td>1x10^6 cells + 1000µM H_2O_2</td>
<td>1x10^6 cells without DCFH-DA</td>
<td></td>
</tr>
<tr>
<td>(positive control)</td>
<td>(positive control)</td>
<td>(negative control)</td>
<td></td>
</tr>
</tbody>
</table>

After 2h in the cell culture incubator the cells were inoculated with *B. vulgatus* mpk (MOI 1) and *E. coli* mpk2 at MOI 1 and MOI 20 as shown in table 5. 500µM and 1000µM H_2O_2 were added to 2 of the wells for positive control and 1 well was left untreated for negative control. 1µg/ml Gentamycin per well was added to prevent bacteria overgrowth. After 1 hour and 16 hours of incubation in the cell culture incubator 7µM DCFH-DA stock solution was added to each well except for the negative control and cells were incubated further for 15min. Afterwards DCs were scraped off the plate, washed twice with PBS and analyzed.
immediately with the BD LSRFortessa II (BD) cell analyzer.

2.7 Proteomics run

Unlabeled shotgun proteome analysis was performed on 6 samples: unstimulated (5min and 16h), *B. vulgatus* mpk stimulated (5min and 16h) and *E. coli* mpk2 stimulated DCs (5min and 16h) by the core proteome facility at the Proteome center, University of Tübingen. Exact protocols for mass spectrometry is available at the proteome facility.

2.8 Bioinformatics analysis and data analysis

The raw-data obtained from the proteome analysis, consisting of 2 biological and 3 technical replicates for each experimental condition, was further assessed by Dr. Marius Codrea (Quantitative Biology Center (QBiC), Tübingen), using bioinformatics tools. Detailed protocol is available in the QBiC center. The process of data analysis is described in detail in the results section (2.3.).
3 Results

3.1 *B. vulgatus* mpk and *E. coli* mpk2 induce different TNFα, IL-6, IL-12p40 and IL-1β secretion profiles.

It has previously been shown that *E. coli* mpk2 stimulated DCs have differences in proinflammatory cytokine secretion profiles in comparison to *B. vulgatus* mpk stimulated DCs (Frick et al., 2006). To assess cytokine production by BMDCs, upon stimulation with pathobiotic (*E. coli* mpk2) and symbiotic (*B. vulgatus* mpk) bacteria, murine DCs were stimulated with viable bacteria at MOI 1 for 16h. 16h samples were restimulated with *E. coli* mpk2 for another 16h. Culture supernatants were collected at time points 16h and 32h and the levels of TNFα, IL-6, IL-12p40 and IL-1β were determined by ELISA (Figure 5).
Results

Figure 6: Cytokine secretion profiles of cultured murine bone marrow derived dendritic cells, isolated from wild type C57BL/6.
Mo: Unstimulated, BV: Stimulated with *B. vulgatus* mpk, EC: Stimulated with *E. coli* mpk2. Stimulation, done 7 days after bone marrow aspiration for 16h, restimulation is done subsequently for another 16h. ELISA is performed with supernatants, collected from both stimulations. The results are representative for three independent experiments. Each experiment was performed in technical triplicates and values represent the mean ± SD of triplicates (*p< 0.05).
Results

*E. coli* stimulation induced a high level secretion of inflammatory cytokines – TNFα (2635pg/ml), IL-6 (59.8ng/ml), IL-12p40 (2510pg/ml) and IL-1β (485pg/ml). Upon restimulation with *E. coli* mpk2 the production of these cytokines strongly decreased, which confirms previous studies, showing desensitization to bacterial challenge (Visintin et al., 2001). Stimulation with *B. vulgatus* mpk resulted in low level secretion of inflammatory cytokines and after restimulation with *E. coli* mpk2 the cytokine production did not increase for TNFα, whereas for IL-6, IL-12p40 and IL-1β there was a slight increase observed, however still much lower than the increase in the restimulated control sample (Mo+EC). The untreated sample showed basal level of secretion of inflammatory cytokines and upon restimulation (time point 32h) the production increased significantly to the level of *E. coli* mpk2 stimulated DCs (time point 16h) for TNFα, IL-12p40 and IL-6. The data suggests that *B. vulgatus* mpk in contrast to *E. coli* mpk2 is less potent to trigger inflammatory responses in DCs and even appears to exert an inhibition of cytokine secretion. Cytokine production is one of the factors that characterizes three
different phenotypic states: non-stimulated, immature DCs (TNFα\textsuperscript{no}, IL-6\textsuperscript{no}, IL-12p40\textsuperscript{no} and IL-1β\textsuperscript{no}), \textit{B. vulgatus} mpk stimulated, semi-mature DCs (TNFα\textsuperscript{low}, IL-6\textsuperscript{low}, 12p40\textsuperscript{low} and IL-1β\textsuperscript{low}) and \textit{E. coli} mpk2 stimulated, fully mature DCs (TNFα\textsuperscript{high}, IL-6\textsuperscript{high}, 12p40\textsuperscript{high} and IL-1β\textsuperscript{high}). These results confirm previous data (Frick et al., 2006) and show the presence of different states of DC maturation, which are used for our proteomics downstream applications.

3.2 \textbf{Stimulation with} \textit{B. vulgatus} mpk \textbf{and} \textit{E. coli} mpk2 \textbf{leads to differential expression of cell surface markers in BMDCs.}

Frick et al. showed that \textit{B. vulgatus} mpk stimulated, semi-mature dendritic cells exhibit a lower percentage of MHC-II and CD40 expression compared to \textit{E. coli} mpk2 stimulated, fully mature dendritic cells. In this experiment unstimulated, \textit{B. vulgatus} mpk stimulated and \textit{E. coli} mpk2 stimulated DCs were analyzed 16h after stimulation for CD11c, MHC-II and CD40 surface expression by flow cytometry (Figure 7). \textit{E. coli} stimulated BMDC express high levels of MHC-II (32,2 \%) and CD40 (79,5\%) and activated and maturated DCs. In contrast, in DCs exposed to \textit{B. vulgatus} mpk, the expression of the two surface
Results

activator markers were nearly unaffected, corresponding to the basal level of expression in unstimulated DCs. This data suggests a reduced ability of *B. vulgatus* mpk to activate and maturate DCs and further characterizes the semi-mature state (low percentage MHC-II and CD40) and the mature state (high percentage MHC-II and CD40) of DCs. Taken together, differential cell surface marker expression and proinflammatory cytokine secretion determine the phenotypical differences of *B. vulgatus* mpk- and *E. coli* mpk2 stimulated DCs, to which we refer as semi-mature and fully mature DCs in the following proteome experiments.
Figure 7: Expression of surface molecules by DCs upon stimulation with commensal bacteria. Unstimulated, *B. vulgatus* mpk stimulated and *E. coli* mpk2 stimulated DCs were double stained with CD11c and either MHC-II (A) or CD40 (B) antibodies and their expression was detected by FACS 16h after stimulation. The numbers indicate the percentage of double positive DCs for each condition.

3.3 Proteome analysis of stimulated DCs with commensal bacteria.

The effect of different bacterial colonization on developing colitis was previously described in mouse models. IL-2−/− mice, which were monocolonized with *E. coli* mpk2 developed colitis, whereas mono-association with *B. vulgatus* mpk, or co-colonization with *E. coli*
Results

mpk2 and *B. vulgatus* mpk did not induce colitis (Waidmann et al., 2003). This finding was further investigated and associated with different DC polarization as a result of the interaction of LP DCs, MLN DCs and BMDCs *in vivo* and *in vitro* with the particular commensal bacteria (Frick et al., 2006; Muller et al., 2008). To get a better understanding of the molecular basis, behind this complex mechanism, BMDCs were stimulated *in vitro* either with *E. coli* mpk2, or *B. vulgatus* mpk, or were untreated for control. Protein samples were collected 5min and 16h after stimulation. A non-labeled shotgun proteome analysis was performed on the samples for protein identification and relative quantitation, giving us the opportunity not only to detect expressed proteins but also to compare their expression levels between the samples at different time points.

3.3.1 Experimental setup

Bone marrow-derived DCs were stimulated either with *B. vulgatus* mpk, or *E. coli* mpk2, or were unstimulated for control. 2 biological and 3 technical replicates for each experimental condition were sent to the proteome center in Tübingen. A shotgun proteome
Results

Analysis was performed and 4559 peptides were identified in total. From those, 1358 peptides were detected in all 6 replicates for all 3 conditions (E. coli mpk2 stimulated, B. vulgatus mpk stimulated and untreated DCs) at both time points 5min and 16h (Table 7, Case 1a). The remaining 3201 peptides were detected in less than 6 replicates for at least one of the conditions. To ensure reproducible protein detection, we required each protein to be detected in at least 4 of 6 replicates for at least one condition, to be considered for further analysis. After removing the proteins, detected in 3 or less replicates, we ended up with 780 proteins that can reliably be analyzed further. Some of them were detected only in one condition and therefore referred to as “state specific” (Table 7, Case 2) and others, in more than one condition, allowing pairwise comparison between the conditions using Student t-test (Table 7, Case 1b).
### Table 7: Description of different cases and statistical tests used in data analysis

<table>
<thead>
<tr>
<th>Case 1</th>
<th>Definition</th>
<th>Statistical tests used</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Proteins, which were detected in all 6 replicates, all 3 conditions and both time points 5min and 16h.</td>
<td>ANOVA (Tukey)</td>
<td></td>
</tr>
<tr>
<td>b) Proteins, which were detected in more than one condition, in at least 4 of 6 replicates at time point 16h.</td>
<td>Student T-test</td>
<td></td>
</tr>
</tbody>
</table>

| Case 2 | Proteins, which were detected only in one condition, in at least 4 of 6 replicates. | Ø |

#### 3.3.2 Determining differentially regulated proteins

The mean intensity values for each protein at time points 16h and 5min were used to perform specific statistical tests for each of the cases, described above. Case 1a proteins were pairwise compared among the 3 conditions using ANOVA (Tukey) as a statistical tool, which considers protein intensity values at both time
Results

points. There were 162 proteins with $p < 0.05$ for at least one of the 3 pairs. ANOVA (Tukey) was impossible to use in Case 1b, because of the variable detection of a protein in the different conditions and time points. Therefore a student T-test was performed, comparing pairwise protein intensity values only at time point 16h. All the Case 1b proteins with $p < 0.05$ for at least one of the 3 pairs were considered significant in terms of differential regulation and were used for further analysis. Case 2 proteins were detected only in one condition and thus taken as differentially expressed per default. Altogether, after assessing the data with statistical tools, we were able to filter 254 significant proteins in total, which are going to be used in the further data mining.

3.3.3 Clustering of the differentially regulated proteins

In order to get a general understanding of the data in terms of functional classification, the 254 differentially regulated proteins were uploaded in the DAVID annotation software. DAVID software uses integrated databases and analytical tools to extract biological meaning from large proteome data, discovering functional groups, enriched in the uploaded proteins dataset (Huang
Proteins were automatically annotated at medium classification stringency to diverse biological processes, which were considered as subclusters to constitute 14 major biological clusters (Figure 8).

Figure 8: Distribution of proteins, annotated to major cellular processes, illustrating the clustering of the proteome data with DAVID annotation software.
254 differentially regulated proteins, preselected with statistical tools, were annotated according to Gene Ontology database to diverse biological processes, organized in 14 major biological clusters, shown in different colors. Numbers indicate the distribution in percentage of all significant proteins among the different clusters.
The purpose of this illustration was to provide a general overview of the data in terms of distribution of the 254 differentially regulated proteins over major biological processes. Five functional classes stand out of the data, containing 66\% of all proteins: Immunity/Inflammation (16\%), Stress response (9,5\%), Protein fate (15,9\%), Transcription/Translation (11,2\%) and Metabolism (13,7\%). The remaining 34\% are similarly distributed over 9 other functional clusters.

3.3.4 Detailed visualization of protein expression at multiple levels of detail

Using the annotation results from the DAVID software, described in section 3.3., we aimed to provide another level of detail, showing subclusters of the major biological processes and even single proteins, which are differentially regulated between *E. coli* mpk2 stimulated, mature DCs, *B. vulgatus* mpk stimulated, semi-mature DCs and unstimulated, immature DCs in order to characterize more precisely the different maturation states of a dendritic cell. We chose Voronoi treemaps as the visualization technique of our proteome data (Bernhardt, Michalik, Wollscheid, Volker, & Schmidt, 2013), because
it enables the comparison of expression level differences and patterns in multiple levels of detail, depending on the interests of the researcher. Our data is displayed in 3 layers of increasing detail. The first layer (Figure 9) visualizes 14 major cellular processes on a 2D image, where every polygon represents a process. The second layer (Figure 9) consists of smaller polygons within the big polygons, representing the subprocesses within a major biological process. The third level (Figure 9) shows a complete, highly detailed picture of the data, where each cell corresponds to a single protein. In order to compare protein expression, the ratios of the mean intensity values for each protein at time point 16h were calculated pairwise and averaged to be assigned to a specific color (Figure 9). All pair combinations were considered: *E. coli* mpk2 stimulated DCs versus unstimulated DCs, which should represent the full maturation process (Figure 9A); *B. vulgatus* mpk stimulated DCs versus unstimulated DCs, which should represent the semi-maturation process (Figure 9B) and *B. vulgatus* mpk stimulated versus *E. coli* mpk2 stimulated DCs, which should report the differences
Results

in proteome of semi-mature and fully mature dendritic cells (Figure 9C).
A. *B. vulgatus* mpk stimulated vs unstimulated DCs

Level 1: Major processes
A  \textit{B. vulgatus} mpk stimulated vs unstimulated DCs

Level 2: Subprocesses
A. B. vulgatus mpk stimulated vs unstimulated DCs

Level 3: Single proteins
Results

B  

E. coli mpk2 stimulated vs unstimulated DCs

Level 1: Major processes
Results

B  E. coli mpk2 stimulated vs unstimulated DCs

Level 2: Subprocesses
B  E. coli mpk2 stimulated vs unstimulated DCs

Level 3: Single proteins
C.  
*B. vulgatus* mpk stimulated vs *E. coli* mpk2 stimulated DCs

Level 1: Major processes
Results

C  B. vulgatus mpk stimulated vs E. coli mpk2 stimulated DCs

Level 2: Subprocesses
Results

C $B. vulgatus$ mpk stimulated vs $E. coli$ mpk2 stimulated DCs

Figure 9: Differential protein expression of distinct DC maturation states, visualized by Voronoi treemaps.
The proteome data is presented as color 2D images at different levels of detail: major functional processes (represented by the biggest polygons); subprocesses (smaller polygons within the biggest polygons) and single proteins (represented by the smallest polygons). Protein expression differences were calculated in a pairwise manner using the mean intensity values for each protein to compare between the three conditions, considering all the possible combinations: (A) $B. vulgatus$ mpk stimulated
Results

DCs vs control; (B) *E. coli* mpk2 stimulated DCs vs control and (C) *B. vulgatus* mpk stimulated vs *E. coli* mpk2 stimulated DCs. Color represents expression level differences. Red: increased expression, Blue: decreased expression, Light grey: No change in expression, Dark grey: not detected in the particular pair. Color intensity is adjusted to the expression level difference on log2 scale. Brighter colors indicate small, darker colors - strong expression level differences.

The visualization with Voronoi treemaps provides a general look on affected cellular processes as a starting point for the analysis of our shotgun proteome results. As expected, upon stimulation with bacteria, a dynamic regulation is seen between different dendritic cell maturation states. In general, a total of 206 proteins are regulated during semi-maturation (69 up- and 137 downregulated) (Figure 9A), 207 proteins are regulated during full-maturation (90 up- and 117 downregulated) (Figure 9B), and 182 proteins (78 up- and 104 downregulated) are differentially regulated between semi-mature and fully mature DCs (Figure 9C). On a more specific scale, in *B. vulgatus* stimulated DCs compared to control, we see a strong downregulation in most of the major biological clusters. An exception is the cluster Apoptosis, where all 3 subclusters are upregulated in *B. vulgatus* stimulated DCs. Furthermore, it is important to
mention, that there are many subprocesses in light grey, especially in the cluster Immunity/Inflammation, meaning that, on average, there is little to no change in protein expression in this major biological cluster, compared to the unstimulated control DCs. However, in Immunity/Inflammation, there is differential regulation to some extent in single subprocesses. Subclusters such as “leukocyte activation”, “T-cell differentiation” and “regulation of T-cell activation” are downregulated, whereas “inflammatoty response”, “regulation of cytokine production”, “regulation of leukocyte mediated immunity” “antigen processing and presentation” are upregulated in *B. vulgatus* stimulated DCs. Additionally, several important clusters, belonging to Migration such as “chemotaxis” and “leukocyte migration are also upregulated in *B. vulgatus* stimulated DCs compared to control. Taken together, our results confirm previous statements, that *B. vulgatus* stimulated DCs are a distinct type of DCs, referred to as “semi-mature DCs”, showing that they are different from unstimulated BMDCs. Furthermore, our results suggest, that semi-mature DCs have a decreased ability to activate immune cells, however
they may initiate a mild pathogenic response, characterized with cytokine production, antigen processing and presentation, as well as increased migration. In contrast to *B. vulgatus* stimulated DCs, *E. coli* stimulated DCs show a strong upregulation of most major biological processes, compared to control. This upregulation is striking for the cluster “Immunity/Inflammation”, where almost every subprocess within the big process is upregulated. The majority of subprocesses related to “Stress response”, “Migration” and “Apoptosis” are also strongly upregulated. Collectively, our observations lead to the conclusion, that *E. coli* stimulated DCs induce full maturation of BMDCs, characterized by higher expression of proteins related to inflammation, cell stress, apoptosis and migration compared to immature DCs, consistent with previous observations and confirming the ELISA and FACS results described above. The last Voronoi treemap shows a direct comparison between *B. vulgatus* stimulated, semi-mature DCs and *E. coli* stimulated fully mature DCs. There is a widespread downregulation observed in the cluster Immunity/Inflammation for *B. vulgatus* stimulated
semi-mature DCs. Immunity cluster is of special importance to us, since it harbors important proteins with potent immunoregulatory effects such as IL-1α, IL1-β, IL-12p40, CD40, NDRG1 and CCL5, all of which are downregulated in *B. vulgatus* stimulated DCs. Noteworthy is the fact, that “response to cellular stress” and “response to DNA-damage” are upregulated in *B. vulgatus* stimulated DCs, despite the low induction of inflammation. This seemingly contradictory finding is further discussed in the following subsection, where we investigate the “stress response” cluster in detail. Of particular interest to us are anti-stress proteins with potential protective properties, such as Phb-1 and TGFβ, which are both upregulated in *B. vulgatus* stimulated DCs. Furthermore, “proteolysis” and “regulation of transcription” are downregulated, whereas other subclusters, which belong to “Transcription/Translation”, “Chromosome organisation”, “Protein fate” and “Apoptosis” are upregulated in *B. vulgatus* stimulated semi-mature DCs. Upon a detailed look at single proteins, an upregulation of Akt1 is to be mentioned as an important kinase, involved in many subprocesses, including cell
growth and differentiation, transcription, translation and cell cycle progression. Our results show that the phenotypical differences observed between *B. vulgatus* and *E. coli* stimulated samples are reflected on a proteome level as well. *B. vulgatus* stimulation results in global downregulation of major pro-inflammatory factors, as well as upregulation of protective factors and proteins with tolerogenic properties. Our results provide a multilayered, detailed picture of protein expression patterns that are specific for semi-mature and fully mature DCs and crucial for understanding their functional and phenotypical differences.

### 3.4 Semi-mature DCs show higher expression of antioxidant and cytoprotective proteins, compared to fully mature dendritic cells.

After creating an overview picture of the whole data we took a closer look into single clusters, aiming to find anti-inflammatory proteins, which are differentially expressed in *B. vulgatus* stimulated DCs in comparison to *E. coli* mpk2 stimulated DCs. We focused on the differences in proteins clustered in stress response, comparing their expression between *B. vulgatus*
stimulated and *E. coli* stimulated cells 16h after stimulation (see Figure 10).
**Figure 10: Expression ratios of proteins, involved in stress response, in comparison between semi-mature and fully mature DCs.**

(A) Ratios of mean intensity values were calculated at time point 16h for stress response proteins, detected in *B. vulgatus* stimulated, semi-mature DCs (*B.v.*) and *E. coli* stimulated, fully mature DCs (*E.c.*). All the proteins in red are expressed in higher levels in fully mature DCs, proteins in blue are expressed in higher levels in semi-mature DCs. (B) Proteins, expressed only in *E. coli* stimulated mature dendritic cells (red) or only in *B. vulgatus* stimulated semi-mature DCs (blue).

<table>
<thead>
<tr>
<th>State specific proteins</th>
<th>Expressed only in <em>E. coli</em> stimulated DCs</th>
<th>Expressed only in <em>B. vulgatus</em> stimulated DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp70-1</td>
<td></td>
<td>Glns</td>
</tr>
<tr>
<td>Arg1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stat5b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ifi75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccl5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mcl1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

Our proteome results indicate a higher expression of proteins belonging to the stress response cluster in *E. coli* stimulated DCs than in *B. vulgatus* mpk stimulated DCs. State specific proteins, shown in the table above (Figure 10B) contribute to this statement as well, given the number of single proteins exclusively expressed in *E. coli*- mpk2 compared to *B. vulgatus* mpk stimulated DCs. On one hand, in the presence of *E. coli*, dendritic cells start to produce high amounts of inflammatory cytokines such as IL-1α, IL-1β. This finding is in line with the ELISA results (see Figure 6), obtained previously. Furthermore, Stat 5a, Stat 5b, Hsp-70, Myd88, CD14, CD44, CD180, HLA-I, Aif and Casp1, which are well known proteins, involved in inflammation, cell damage and cell death, are upregulated in *E. coli* stimulated DCs. On the other hand, *B. vulgatus* stimulated, semi-mature dendritic cells express a high amount of proteins related to protection from oxidative stress e.g. Prohibitin (Phb-1), Superoxide dismutase (Sod1), Glutathione-S-Transferase omega-1 (Gsto1), Thioredoxin (Txn), Glutamate-cysteine ligase (Gclm). Moreover, proteins involved in a tolerogenic DC response e.g. TGFβ are upregulated in semi-mature DCs.
This data confirms the potential of *E. coli* to induce a strong inflammatory stress response and cell death in BMDCs, whereas *B. vulgatus* stimulated DCs appear to be able to induce peripheral tolerance via TGFβ and to deal better with stress, in particular with reactive oxygen species (ROS), indicated by increased production of antioxidant enzymes.

**3.5 Prohibitin (Phb1) amount is slightly increased in *B. vulgatus* mpk stimulated samples, compared to *E. coli* mpk2 stimulated samples.**

Based on a previous study (Theiss, Vijay-Kumar, et al., 2009) we selected Phb-1 as an important candidate to validate, as it is known to play an important role in ROS metabolism. It acts as a modulating protein to promote Nrf2 accumulation in the nucleus and thus production of AREs (Antioxidant response elements), which are highly expressed in *B. vulgatus* mpk stimulated DCs i.e. Sod1, Gsto1, Gclm (see Figure 10A). To assess the amount of Phb-1 produced, western blotting was performed and compared with proteome data (Figure 11).
Results

Figure 11: Prohibitin expression of semi-mature and fully mature DCs.
Western blot results on Prohibitin expression in *B. vulgatus* mpk stimulated (B.v.) and *E. coli* mpk2 stimulated (E.c.) bone marrow derived dendritic cells 16h (16”) after stimulation. The house-keeping protein expression of β-actin was included as loading control. (B) Proteome results for Prohibitin in *B. vulgatus* mpk stimulated and *E. coli* mpk2 stimulated samples at time point 16h, illustrated by bar graph using the mean intensity values of the protein, obtained from the proteome data. *E. coli* mpk2 stimulated DCs show low expression of the antioxidant protein in both cases.

As the differences between the samples were too subtle to detect by eye, optical band density values were measured with ImageJ software and Phb1 expression was normalized according to the values of the house-keeping protein β-actin (Figure 11A). Results were compared to the intensity values for Prohibitin, obtained from the mass spectrometric reads (Figure 11B). The comparison of western blot data with proteome data shows a high degree of conformity in terms of expression values. In both western blot data and proteome data, semi-mature DCs
show a slightly higher amount of the antioxidant Prohibitin at time point 16h, suggesting a better protection against oxidative substances in comparison to fully mature DCs. We acknowledge that the expression level difference we observed is very small, however for key regulatory molecules such as Phb1, such small differences can have a bigger impact on downstream pathways.

3.6 DCs stimulated with pathobiotic bacteria harbor increased amounts of ROS, compared to DCs stimulated with symbiotic, protective bacteria.

As it is known that ROS imbalance plays an important role in inflammatory processes and in particular colitis (A. Wang et al., 2014), we aimed at investigating ROS production in DCs upon stimulation with pathobiotic and symbiotic bacteria. Viable BMDCs were stimulated with *B. vulgatus mpk* at MOI 1 and *E. coli mpk2* at MOI 1 and MOI 20 for 1h and 16h. DCFH-DA reacts with ROS to produce the fluorescent DCF, which was measured by FACS using the FITC-channel, 1h (Figure 12A) and 16h (Figure 12B) after stimulation. The level of DCF corresponds to the amount of ROS in the cells.
Results

Figure 12: Production of reactive oxygen species (ROS) upon stimulation with commensal bacteria.

ROS amount in the cytoplasm was measured with FACS in unstimulated, *B. vulgatus* stimulated (MOI 1) and *E. coli* stimulated (MOI 1 and MOI 20) bone marrow derived dendritic cells 1h (A) and 16h (B) after stimulation, using DCFH-DA as a fluorescence marker. (C) Untreated DCs were used as negative control and two positive controls with 500µM and 1000µM H2O2 were also included. Representative histograms are shown from 2 independent experiments. DCF signal intensity, which corresponds to ROS amount in the cytoplasm, is captured on the FITC channel.
Results

The negative control showed negligible to no amount of ROS (109) and increasing amounts of H$_2$O$_2$ in the positive controls gave rise to increased FITC-signals, suggesting that the assay is reliable in quantifying ROS levels. Immature DCs, stimulated with *E. coli* showed the highest amount of ROS at both time points. Whereas at time point 1h there was a minimal difference between MOI 1 and MOI 20, at time point 16h the ROS production by DCs stimulated with *E. coli* mpk2 at MOI 20 was considerably higher, indicated by a high mean fluorescence value (2399). *B. vulgatus* stimulated DCs showed slight increase of ROS (551), nevertheless still close to the basal level of the unstimulated sample (402). Over time a slight increase in the mean fluorescence intensity in both unstimulated and *B. vulgatus* stimulated DCs was observed but not as high as the increase in *E. coli* mpk2 stimulated DCs at MOI 1 and 20. In conclusion, we demonstrated that *E. coli* challenge results in higher amount of ROS accumulation in the DC cytoplasm, compared to stimulation with the symbiotic *B. vulgatus*. 


3.7 *E. coli* stimulation of BMDCs leads to higher surface expression of the migration marker CCR7.

Stimulation with *E. coli in vivo* induces increased DC migration towards lymph node, whereas *B. vulgatus* stimulated DCs showed reduced ability to migrate (Muller et al., 2008). To assess DC migration upon stimulation with pathobiotic *E. coli* mpk2 and symbiotic *B. vulgatus*, BMDCs were inoculated with viable bacteria at MOI 1 for 16h. Surface expression of chemokine receptor CCR7 was measured by FACS (Figure 13).
Results

Figure 13: Expression of CCR7 by DCs upon stimulation with commensal bacteria.
FACS analysis of CCR7 expression in unstimulated, *B. vulgatus* stimulated (MOI 1) and *E. coli* stimulated (MOI 1) bone marrow derived dendritic cells at time point 16h. Cells are double stained with CCR7 and a specific DC-marker - CD11c. The percentage of double positives cells (> $10^3$ on the x-axis and >$10^3$ on the y-axis) in each condition is shown at the bottom right corner of the corresponding graph. *E. coli* stimulated DCs demonstrate the highest CCR7 expression.

*E. coli* mpk2 stimulated DCs showed increased surface expression of the migration marker CCR7, indicated by population shift and population size (89,6%). On the other hand, stimulation with *B. vulgatus* mpk did not lead to a major increase in CCR7 expression. CCR7 amount on the cell surface of *B. vulgatus* mpk stimulated DCs was more close to that of unstimulated control samples (56.6% vs 42.5%). Additionally, our proteome results indicate upregulation of migration related proteins in *E. coli* mpk2 stimulated mature DCs, compared to *B. vulgatus* mpk
Results

stimulated DCs such as Fn1, Actn4, Myh9, Coro1, Rtn4 (Figure 14). In our proteome experiment, CCR7 and CCL5 were detected only in E. coli mpk2 stimulated DCs and not in the other two conditions. Important for us was the regulation of CCR7, as it is a crucial chemokine for DCs migration and it was previously shown to be upregulated in fully-mature DCs, compared to semi-mature DCs (Muller et al., 2008). Taken together, our proteome data and FACS results suggest a reduced potential of B. vulgatus mpk to induce migration in immature DCs, compared to E. coli mpk2.
Figure 14: Expression ratio of proteins, involved in migration, in comparison between semi-mature and fully mature DCs.

Ratios of mean intensity values at time point 16h were calculated for proteins detected in *B. vulgatus* mpk stimulated semi-mature DCs (*B.v.*) and *E. coli* mpk2 stimulated fully mature DCs (*E.c.*). The proteins below 1 (red) are more expressed in fully mature DCs, those above 1 (blue) are more expressed in semi-mature DCs. Expression of migration related proteins is strongly increased in *E. coli* mpk2 stimulated DCs, compared to *B. vulgatus* mpk stimulated DCs. CCR7 and CCL5 were detected only in *E. coli* mpk2 stimulated mature dendritic cells.
4 Discussion

The main findings of this study are:

(i) *E. coli* has strong inflammation inducing potential, whereas *B. vulgatus* shows inflammation suppressive properties, mediated by inhibition of cytokine secretion.

(ii) *B. vulgatus*, compared to *E. coli* has reduced ability to activate and maturate DCs, characterizing two distinct DC maturation states: semi-mature state (TNFα\text{low}, IL-6\text{low}, IL-12p40\text{low} and IL-1β\text{low}; low percentage MHC-II\text{\textsuperscript{+}} and CD40\text{\textsuperscript{+}} cells) and mature state (TNFα\text{high}, IL-6\text{high}, IL-12p40\text{high} and IL-1β\text{high}; high percentage MHC-II\text{\textsuperscript{+}} and CD40\text{\textsuperscript{+}} cells).

(iii) Colitogenic *E. coli* demonstrates higher potential to induce inflammation and migration in DCs, whereas *B. vulgatus* stimulated DCs exert a regulatory and dampening effect on inflammation and migration.
(iv) Colitogenic *E. coli* stimulation leads to increased accumulation of ROS in BMDCs, an effect not seen in *B. vulgatus* stimulated samples.

(v) Semi-mature dendritic cells appear to have activated countermeasures to oxidative stress, as members of Keap1-Nrf2 pathway are upregulated.

(vi) Western blot results show that *E. coli* stimulated samples, compared to *B. vulgatus* stimulated and control samples, express less Phb1 in line with our proteome results.

In our study we investigate the effect of *B. vulgatus mpk* and *E. coli mpk2* on the activation and maturation of DCs, the major antigen-presenting cells in the gut. Furthermore, we are interested in defining specific protein expression patterns, which characterize different maturation states of DCs. Finally, we are curious about how these maturation states affect and modulate intestinal inflammation in IBD and what parallels can we draw from our results to the clinical observations.
The two gram-negative commensal bacteria we used to stimulate BMDCs have already been shown to have different influences on gut homeostasis and developing colitis in mammalians. In HLA-B27 transgenic rats, *B. vulgatus* induced severe colitis, while *E. coli* did not (Rath, Wilson, & Sartor, 1999). In contrast, our group showed that in IL-2-deficient mice kept under SPF conditions monocolonization with *E. coli* led to colitis, whereas *B. vulgatus* monocolonization did not lead to disease. Interestingly, co-colonization with *E. coli* and *B. vulgatus* did not lead to colitis as well, suggesting that *B. vulgatus* has the potential to protect from developing the disease (Waidmann et al., 2003). This contradiction between the two research groups can stem from various crucial differences in experimental setting. Most importantly, the experiments are performed on two different species (mouse vs rat) using different strains of the same bacteria (e.g. *B. vulgatus* vs *B. vulgatus* mpk). The *B. vulgatus* strain, used by Rath et al. was received as a gift from another group that isolated it from a guinea pig with carrageenan-induced colitis. In comparison, Waidmann et al. performed the *in vivo* experiments in
germ free (GF) mice with mass spectrometry proven *E. coli* mpk2 and *B. vulgatus* mpk. Equally important is the environment the animals were kept in and differences between animal facility of rat and mice, as well as substantial differences of gut microflora between these animals.

As dendritic cells are an important link between microbiota and adaptive immune system, it was interesting to see how gut commensal bacteria interact with them. Frick et al. (2006) found that *in vitro* stimulation of BMDCs with *B. vulgatus* mpk leads to non-responsiveness of the cells to further *E. coli* stimulus, indicated by reduced TNFα and IFNγ secretion and reduced Th1 polarization, compared to BMDCs, stimulated only with *E. coli*. They argued that *B. vulgatus* is able to drive a DC into a specific state, non-responsive to a second stimulus, which was referred to as “semi-mature state”. It was further characterized by low secretion of inflammatory cytokines and low expression of surface activation markers. In contrast, stimulation with *E. coli* mpk2 led to activation and full maturation of DCs, characterized by high production of inflammatory cytokines and significant
increase in DC surface activation marker expression, such as CD40 and MHC-II. Moreover, fully mature DCs showed increased migration towards MLN, where they were capable of polarizing TH cells into TH1 direction (Frick et al., 2006; Muller et al., 2008). These different phenotypes of DCs have already been described and termed previously in the literature and were thought to play an integral role in maintaining tolerance or immunity in the mice gut (Lutz & Schuler, 2002).

Our FACS and ELISA results correspond to these findings confirming the presence of three different phenotypical states in our samples: immature DCs (TNFα\textsuperscript{no}, IL-6\textsuperscript{no}, IL-12p40\textsuperscript{no} and IL-1β\textsuperscript{no}; low percentage MHC-II\textsuperscript{+} and CD40\textsuperscript{+}), semi-mature DCs (TNFα\textsuperscript{low}, IL-6\textsuperscript{low}, IL-12p40\textsuperscript{low} and IL-1β\textsuperscript{low}; low percentage MHC-II\textsuperscript{+} and CD40\textsuperscript{+}) and fully mature DCs (TNFα\textsuperscript{high}, IL-6\textsuperscript{high}, IL-12p40\textsuperscript{high} and IL-1β\textsuperscript{high}; high percentage MHC-II\textsuperscript{+} and CD40\textsuperscript{+}). We chose to use bone marrow-derived dendritic cells (BMDCs) in our experiments, because it is a well-established system to study DCs \textit{in vitro}. Only cell cultures with CD11c\textsuperscript{+} cell abundance more than 80\% were considered for experiments. Further purification of the
cells with cell sorting methods i.e. magnetic sorting and FACS sorting were tried, but were unsuitable, because the method itself stresses and maturates DCs.

After performing a shotgun proteome analysis, three different pairwise comparisons were made (i.e. *B. vulgatus* mpk stimulated vs. unstimulated DCs; *E. coli* mpk2 stimulated vs. unstimulated DCs and *B. vulgatus* mpk stimulated vs. *E. coli* mpk2 stimulated DCs). Differences in protein expression patterns were illustrated by Voronoi treemaps in multiple levels of detail. Looking at the first and second levels, a researcher can have an overview of the data. If there are many upregulated or downregulated subclusters in one major biological process (e.g. Immunity/Inflammation), we would spotlight this major cluster as potentially characteristic for one of the DC maturation states. After determining the focus, looking at the overview layers, a researcher can now have a look at the third and last level, which provides the most detailed picture of our data. At this layer, expression levels of each protein can be seen explicitly and it is possible to draw tentative hypotheses using such data, however one should be wary and consider the interactions with other proteins.
to determine the exact role of a protein in that particular cluster. Akt1, for instance, is seen in the proteome data as upregulated in *B. vulgatus mpk* stimulated DCs. It is a multifunctional kinase, which plays a role in numerous biological processes e.g. Growth & Glucose metabolism, Cell cycle & survival, Protein synthesis, etc. (see Figure 15). It may inhibit or stimulate other proteins and thus have a different influence on major biological functions. Hence, to determine the exact role of a protein in DC maturation, it must be kept in mind that proteins have diverse roles in different pathways and that they can interact with each other in an unpredictable manner. This information cannot be represented in our Voronoi treemaps, therefore more analysis on a pathway level should be performed, together with the confirmation of outcomes.
Discussion

Figure 15: Akt1 signaling pathway
The image is adopted from (Emamian, 2012). An example for the variety of different functions and interactions a protein can have.

Our proteome catalogue represents an important starting point in the data mining of this proteome experiment. With the guidance of our visualization, we focus on clusters and subclusters that are particularly relevant to us and analyze them further.
One of the clusters we focused on was “Immunity/Inflammation”, because it is one of the most differentially regulated clusters between the 3 pairwise comparisons. All subclusters, belonging to this major process are upregulated in E. coli stimulated DCs, compared to unstimulated and B. vulgatus mpk stimulated DC. Hence, “Immunity/Inflammation” is a distinctive cluster for mature DCs and therefore a good candidate for further investigation. Moreover, this cluster harbors important proteins which are capable of modulating intestinal inflammation. Pro-inflammatory proteins i.e. IL1-β, IL-12p40, CD40 were upregulated in E. coli stimulated mature DCs compared to immature and semi-mature DCs. IL1-β and IL-12p40 as well as CD40 were previously shown in our results to be highly expressed upon maturation of DCs using ELISA (Figure 6) and FACS (Figure 7). Furthermore, these proteins are very well known in the literature for their pro-inflammatory properties. CD40 is a cell surface protein known to be involved in the maturation of DCs and in inflammation processes. Its function has been thoroughly discussed in the review by (Ma & Clark, 2009). IL-12p40 is a pro-
inflammatory cytokine, secreted from APCs i.e. DCs, which is believed to induce pathogenic T-helper cells polarization and tissue inflammation in response to microbial components (Krummen et al., 2010). It plays a crucial role in various autoimmune diseases, including inflammatory bowel disease (Croxford, Kulig, & Becher, 2014; Fuss et al., 2006; Gee, Guzzo, Che Mat, Ma, & Kumar, 2009; Parronchi et al., 1997). Another important mediator of inflammation and host response to infection is IL-1β. It is reported to be released by DCs, monocytes or macrophages after stimulation with pathogen-associated molecular pattern molecules (PAMPs) or damage-associated molecular pattern molecules (DAMPs) (Lotze et al., 2007). The interaction with a pathogen activates Caspase-1, which cleaves the inactive pro-IL-1β to be subsequently released into the extracellular matrix as biologically active IL-1β (Eder, 2009). Strong interaction with the inflammasome as a mechanism for induction of IL-1β secretion has been reported by many researchers (Aguilera, Darby, & Melgar, 2014; Church et al., 2008). Moreover, IL-1β has been shown to be a pro-inflammatory cytokine in many diseases including IBD and various
autoimmune disorders (Dinarello, 1996; Gionchetti et al., 1992). All those aforementioned proteins are detected in our proteome experiment (Table 6). IL-1α, IL-1β and Casp1 are upregulated in *E. coli* stimulated mature DCs compared to *B. vulgatus* mpk stimulated semi-mature DCs, suggesting that *E. coli* mpk2 is capable of inducing an inflammatory response via stimulation of the inflammasome. We argue that activation of the inflammasome may play an important role in DC-maturation process and tissue inflammation in IBD.

Another central factor of inflammation is the TLR signaling system. TLRs represent a key mechanism of DCs to sense pathogens and initiate appropriate immune response (Akira, Uematsu, & Takeuchi, 2006; Medzhitov et al., 1997). As LPS is a vital component of the outer membrane of Gram-negative bacteria (i.e. *E. coli* and *B. vulgatus*), an interaction with TLR-4 takes place. LPS/TLR4 downstream signaling can be divided in MyD88-dependent and MyD88-independent pathway (Lu et al., 2008). Activation of the first triggers series of events, including NFκB release, which leads to the transcription of multiple pro-inflammatory genes.
including the cytokines mentioned before (i.e. IL1-β, IL-12p40) (O'Neill, 2006). Furthermore, LPS recognition is promoted by LBP and CD14 (O'Neill, 2006). MyD88 and CD14 were detected in our proteome experiment and shown to be upregulated in *E. coli* stimulated mature DCs, compared to immature and semi-mature DCs. Together with the upregulated pro-inflammatory cytokines, we show that activation of TLR4 via MyD88 is a distinctive feature of mature DCs in response to a potent pathogenic organism.

Furthermore, using our proteomics catalogue, researchers can search for single proteins which are less investigated in the context of DC differentiation and intestinal inflammation. Ship1 (SH2 domain-containing inositol 5'-phosphatase 1), which is upregulated in *B. vulgatus* stimulated DCs, has been recently shown to protect from colitis (Fernandes, Iyer, & Kerr, 2013) but has never been related to the maturation processes of DCs. C/EBPβ (CCAAT-enhancer-binding protein β), another less known protein, which is upregulated in *B. vulgatus* stimulated DCs, is a transcription factor responsible for the secretion of various cytokines. However its involvement
in DC maturation and relevance to inflammatory diseases have not been studied well. Our data can be used as a starting point for discovering such new proteins, which may play a crucial role in DC maturation or inflammation.

The next focus of our study was placed on processes related to migration. This major biological function of DCs is very well studied in the context of inflammation and DC maturation process (Alvarez et al., 2008; Johnson & Jackson, 2014; Stock, Napolitani, & Cerundolo, 2013; Teijeira et al., 2014). Our proteome data and FACS analysis of CCR7 indicate higher migratory capability of *E. coli* mpk2 stimulated, fully mature DCs, in line with aforementioned studies and Muller et al. (2008). Subclusters and single proteins (e.g. Fn1, Actn4, Myh9, Coro1 and Rtn4), included in the migration cluster are upregulated in *E. coli* stimulated DCs compared to *B. vulgatus* mpk stimulated and unstimulated DCs. Furthermore, the surface expression of CCR7, detected by FACS, has significantly increased after stimulation with *E. coli* mpk2. *B. vulgatus* mpk did not remarkably increase CCR7 expression, confirming previous data (Muller et al., 2008). In our proteomics experiment, CCR7 is detected
only in *E. coli* mpk2 stimulated DCs. It may be due to the fact that its abundance in semi-mature and immature DCs was too low to be detected by the mass spectrometer. CCR7 is a crucial chemokine for the controlled migration properties of DCs. Without this chemokine, DCs are unable to find their way through lymphatic vessels and to enter regional lymph nodes (Forster et al., 1999; Martín-Fontecha et al., 2003; Saban, 2014). DC mobilization signals, including upregulation of CCR7, are shown to be induced primarily in the presence of pro-inflammatory cytokines. The importance of IL-1β and TNFα for DC migration has been indisputably demonstrated in experiments, by neutralizing them (Cumberbatch & Kimber, 1995) and in mice deficient for TNFα type II receptors (Cumberbatch & Kimber, 1995), IL-1 type 1 receptor (Cumberbatch, Dearman, & Kimber, 1999) and caspase-1 (Antonopoulos et al., 2001). The mechanism(s), by which these inflammatory cytokines induce migration include altered expression of adhesion molecules and chemokine receptors (e.g. CCR7) on DCs. All aforementioned cytokines were shown to be upregulated in fully mature DCs in the results of our proteomics
experiment. On the other hand, it has been shown that DCs can migrate in the absence of inflammatory stimuli (Brand, Hunziker, & Braathen, 1992; Bujdoso, Hopkins, Dutia, Young, & McConnell, 1989; Tomura et al., 2008), however signals that induce this “spontaneous” migration remain cryptic. Assessing single proteins belonging to migration cluster may give more insights on this issue. So far in our proteome analyses we have focused on two main issues. First, in our data and our experimental setting we have confirmed the presence and the regulation of inflammation/migration related factors that have been consistently described in the literature. Second, we have provided data on many proteins that are differentially expressed in distinct DC phenotypes, which are not investigated so far in a DC maturation/IBD context. These proteins can be the focus of further efforts aiming at clarifying their roles in IBD.

Our next focus were the proteins included in the stress response cluster. Many of the proteins we have discussed before in the context of inflammation are also present in this cluster, due to multiplicity of protein functions and overlapping processes between stress
response and inflammation. Nevertheless, there were several subclusters related to protection from stress, which were upregulated in B. vulgatus mpk stimulated DCs. Expression patterns, belonging to these subclusters were of specific importance for us, since these patterns may be characteristic for the semi-mature state of DCs. A closer look at individual proteins revealed upregulation of TGFβ in B. vulgatus mpk stimulated DCs. There is an increasing body of evidence that TGFβ secretion of DCs plays a major role in the development of Foxp3+ T_{reg} cells and induction of tolerance (Belkaid & Oldenhove, 2008; Coombes et al., 2007; Coquerelle & Moser, 2008; Li, Wan, Sanjabi, Robertson, & Flavell, 2006). Many types of APCs are capable to convert naïve T-cells to Foxp3+ T_{reg} cells, however DCs appear to be more efficient (Yamazaki et al., 2003). It is also reported that the status of activation of dendritic cells has a different influence on their capacity to induce T_{reg} response (Belkaid & Oldenhove, 2008; Bettelli et al., 2006; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006). TGFβ deficiency has been shown to induce early autoimmune diseases and IBD in mice, indicating that this cytokine plays a major role in
preventing autoimmune responses (Kulkarni et al., 1993; Shull et al., 1992). Altogether, this regulatory cytokine has been shown to have a diverse effect on the immune system in terms of maintaining tolerance and would be an important candidate for further investigation, as its upregulation may be a distinctive feature of semi-mature DCs. Intriguingly, searching for other important candidates, upregulated in B. vulgatus mpk stimulated DCs, we discovered several other proteins, related to protection from oxidative stress. In this thesis, we chose to further investigate the involvement of these proteins in IBD.

Oxidative stress is thought to be one of the leading causes of intestinal inflammation (Buffinton & Doe, 1995; D'Odorico et al., 2001; Keshavarzian, Morgan, Sedghi, Gordon, & Doria, 1990; Keshavarzian et al., 1992; Koutroubakis et al., 2004; Kruidenier, Kuiper, Lamers, & Verspaget, 2003; Kruidenier, Kuiper, Van Duijn, et al., 2003; Lih-Brody et al., 1996). We were able to show that E. coli stimulated fully mature DCs harbor more ROS than B. vulgatus stimulated, semi-mature and unstimulated, immature DCs. As the adverse effects of excessive ROS
production is well documented, high amounts of ROS combined with impaired stress response mechanisms we observed (discussed in the following paragraph) can be detrimental for the organism. ROS overproduction can cause severe damage to vital molecules such as lipids, carbohydrates, proteins and DNA and thus influence main functions of the cell. DNA lesions, caused by the interaction with oxidative molecules, have been shown to be relevant to mutagenesis (Hussain et al., 2000) and eventually lead to intestinal tumorigenesis (D’Inca et al., 2004; Dincer et al., 2007; Ferguson, 2010; Meira et al., 2008). Lipid oxidation end-products, such as 4-hydroxynonenal (HNE) and oxysterols have been shown to be crucial for developing chronic inflammation, as aldehydes and cholesterol are the main substrates for prostaglandins (PGs) and for leukotriene (LT) formation (Mascia et al., 2010; Nair et al., 2006; Rezaie, Parker, & Abdollahi, 2007). Based on this data, it is tempting to speculate that the observed redox imbalance is one of the driving factors for the phenotypical differences we observed between semi-mature and fully mature DCs. It has been demonstrated that ROS can directly stimulate
secretion of inflammatory cytokines via activation of NFκB and other inflammatory pathways (Asehnowe, Strassheim, Mitra, Kim, & Abraham, 2004; Cruz et al., 2007). Direct and indirect activation of the inflammasome by ROS has also been reported (Dostert et al., 2008; Martinon, 2010).

In addition, free radicals can cause disruption of the mucosal barrier, characterized by increased permeability and impaired wound healing (Banan et al., 2000; A. Wang et al., 2014). Disintegration of the colonic epithelium, caused by ROS, has already been shown in CD patients in quiescent stage (Rao, Baker, Baker, Gupta, & Holycross, 1997). Furthermore, 40% of the first-degree relatives have increased intestinal permeability without any inflammation signs (Buhner et al., 2006; Fries et al., 2005). Once the mucosal barrier is damaged, pathogenic bacteria can infiltrate the sterile submucosa and initiate numerous destructive cascades of immune response (Banan et al., 2000; Fasano & Shea-Donohue, 2005). Besides local tissue damage, impaired intestinal barrier function, referred to as “leaky gut syndrome” has been linked to the development of extraintestinal diseases, including
multiple sclerosis, ankylosing spondylitis, asthma, type 1 diabetes, autoimmune hepatitis (Fasano, 2012; Lin, Zhou, Zhang, & Wang, 2015). The etiology of the leaky gut is poorly understood, however it has been associated with loosening of intestinal epithelial tight junctions in a zonulin dependent manner. (Fasano et al., 2000; W. Wang, Uzzau, Goldblum, & Fasano, 2000). This may lead to increased paracellular passage of antigens, represented by food components (e.g. gluten in celiac disease) or bacterial epitopes. The abnormal antigen delivery to the well vascularized submucosa may trigger a strong immune reaction, which increases the chances for an autoimmune process. As ROS is a potential cause of mucosal barrier disruption and thereby the development of leaky gut syndrome, a detailed investigation of the molecular mechanisms behind oxidative stress may be crucial for understanding the origin of IBD and many other chronic diseases.

Numerous studies report that ROS are inducers of inflammation rather than mere byproducts of it. Studies from 1980s show that oxidizing agents are able to induce severe colitis in mice with typical clinical and histological
features of the disease (Bilotta & Waye, 1989; Meyer, Brand, DeLuca, & Spiro, 1981). This was observed after administration of H$_2$O$_2$, used at this time to treat fecal impaction. Another study showed that mice deficient in antioxidant genes (i.e. glutathione peroxidase gene), developed an intestinal inflammation consistent with the symptoms and pathology of IBD (Esworthy et al., 2001). Mice lacking the antioxidant enzyme showed increased ROS abundance which caused a crypt destructive colitis, similar to UC (Esworthy et al., 2001). Although these studies cannot be taken as a definitive basis to prove causality, they strongly indicate that redox imbalance has an etiological role in IBD.

In the Stress response cluster (see Figure 10A) we discovered many antioxidant enzymes as well as a crucial regulatory protein of anti-oxidative cascades, called Prohibitin (Phb1). All of them were differentially regulated upon stimulation with commensal bacteria. Prohibitin was found to be upregulated in B. vulgatus mpk stimulated DCs compared to E. coli mpk2 stimulated DCs. Our Western blot supported this finding, showing slightly increased Phb1 amount in B. vulgatus mpk stimulated
DCs. Phb1 drew our attention, as it has previously been shown to protect against oxidative stress and to attenuate colitis in mice (Theiss et al., 2007; Theiss, Vijay-Kumar, et al., 2009). Additionally, Prohibitin abundance was shown to be decreased in patients with Crohn’s disease as well as in two distinct models of experimental colitis, showing that Phb1 is less expressed under inflammatory conditions. Moreover, *in vitro* experiments showed that in an oxidative environment, Prohibitin expression is reduced as well, but after removing the oxidative agents, it is restored to normal levels (Theiss et al., 2007). Our results extend the reports of previous findings which were performed on colonic epithelial cells *in vitro* and *in vivo*. In our study we analyzed Phb1 expression in distinct DC maturation states. Mature DCs, which were stimulated with the colitogenic *E. coli* mpk2, showed decreased levels of Phb1, similar to the colonic epithelial cells under inflammatory conditions. On the other hand, in our proteome and Western blot experiments, dendritic cells stimulated with the protective *B. vulgatus* mpk showed increased Prohibitin expression. This is an interesting observation as it has been previously shown that
restoration of Prohibitin levels via Phb1 overexpressing transgenic mice or using recombinant adenovirus vectors ameliorates colitis (Theiss, Jenkins, et al., 2009; Theiss et al., 2011). Considering ample research data mentioned above, we propose that Phb1 is a crucial protein for mediating the protective properties of *B. vulgatus* mpk in colitis.

Mechanically, on a pathway level, Phb1 is primarily linked to the positive regulation of Keap1-Nrf2 pathway (see Figure 16), which is one of the most important pathways for fighting excessive oxidative stress (Bocci & Valacchi, 2015; Sykiotis & Bohmann, 2010; Theiss et al., 2007). More details on the mechanism of action of Prohibitin was later provided by (Kathiria, Butcher, Hansen, & Theiss, 2013; Theiss, Jenkins, et al., 2009), showing that Phb1 is able to directly inhibit TNFα induced activation of NFκB and thus to impair the production of inflammatory cytokines. The latter strongly suggests that Phb1 is capable of modulating intestinal inflammation in Nrf2 independent manner as well.
Figure 16: Regulation of Keap1-Nrf2
The image is adopted and modified from Sykiotis and Bohmann (2010). Keap1-Nrf2 pathway is major antioxidant pathway in the cell. Under normal condition the transcription factor Nrf2 is repressed by the Keap1 protein and undergoes targeted degradation in the proteasome. Under oxidative stress (ROS), Keap1 releases Nrf2 to fulfill its role as a transcription factor. Prohibitin (PHB1) and P62 promote Nrf2 accumulation in the nucleus as well and thus the transcription of Antioxidant Response Elements (ARE).

Nrf2 is a transcription factor which is repressed under normal conditions by Keap1. Normally the protein complex has a short half-life in the cytoplasm (20min) and if not used, it is degraded in the proteasome. However, in the presence of oxidants, electrophiles or sulforaphanes, Nrf2 is released and accumulates in the nucleus, where it
is responsible for the transcription of Antioxidant Response Elements (AREs) (Bocci & Valacchi, 2015; Ishii, Itoh, & Yamamoto, 2002; Kensler, Wakabayashi, & Biswal, 2007). Interestingly, after assessing our previously established stress response cluster list (see Figure 10 above), we found 4 major representatives of the Antioxidant Response Elements (i.e. superoxide dismutase (Sod1), glutathione s-transferase omega-1 (Gsto1), thioredoxin (Txn) and glutamate-cysteine ligase (Gclm)). All of them were expressed more in B. vulgatus mpk stimulated DCs compared to unstimulated and E. coli stimulated DCs. Presumably, fighting oxidative stress via activation of Nrf2 is an important feature of semi-mature DCs and that may be a key factor in protection from colitis. Khor et al. (2006) showed that Nrf2-deficient mice are more susceptible to develop DSS-induced colitis than wild type. Furthermore, antioxidants e.g. Sod1, Trx have been shown in multiple studies to attenuate colitis in experimental mice models (Kruidenier, van Meeteren, et al., 2003; Oz, Chen, McClain, & de Villiers, 2005; Segui et al., 2005; Tamaki et al., 2006). Altogether, these findings indicate the importance of this major anti-
oxidative pathway in IBD, which is not surprising as oxidative stress plays a significant role in the pathogenesis of intestinal inflammation (Biasi, Leonarduzzi, Oteiza, & Poli, 2013; Grisham, 1994; Iborra et al., 2011; Kruidenier, Kuiper, Van Duijn, et al., 2003; Kruidenier & Verspaget, 2002; Moret et al., 2014).

Therapeutic applicability of antioxidants or regulatory proteins such as Prohibitin can be further studied in the future, as they may have the potential to ameliorate IBD. Considering the “leaky gut” hypothesis, the use of antioxidants or prohibitin may be beneficial not only for managing intestinal pathologies, but also in treating many other chronic inflammatory and autoimmune conditions as well. Additionally, as *B. vulgatus* mpk is able to protect from excessive ROS production via activation of antioxidant pathways, the therapeutic applicability of probiotics containing *B. vulgatus* mpk, can also be investigated in IBD or other ROS related diseases such as Celiac disease, Type 1 Diabetes, Asthma, Multiple sclerosis, Ankylosing spondylitis.
5 Conclusion

In this study we aimed at gaining more insight into inflammatory bowel disease by investigating the immunological factors mediating it. We used a proteomics approach targeting dendritic cells, stimulated with the symbiotic B. vulgatus mpk or pathobiotic E. coli mpk2. Stimulation with symbiotic bacteria resulted in:

(i) reduced activation and maturation of dendritic cells
(ii) reduced inflammation and migration of dendritic cells
(iii) decreased accumulation of ROS
(iv) activation of countermeasures to oxidative stress

The reduced ability of B. vulgatus mpk to activate and maturate DCs is indicated by inhibition of proinflammatory cytokine secretion i.e. TNFα, IL-6, IL-12p40 and IL-1β and the difference in surface expression markers such as MHC class II and CD40. B. vulgatus mpk stimulated samples show downregulation in proinflammatory pathways and processes, exemplified by
a downregulation of proteins which are part of the TLR4-signalling pathway, formation of the inflammasome and chemokine guided migration. In addition to decreased proinflammatory gene expression, increased expression of the anti-inflammatory protein TGFβ was detected as well, suggesting an involvement of semi-mature DCs in induction of peripheral tolerance. We were also able to show that DCs stimulated with the symbiotic *B. vulgatus* mpk harbor basal ROS amounts, whereas the pathobiotic *E. coli* mpk2 induces high levels of reactive oxygen species in DCs. Additionally, we detected an increase of the antioxidant capacity in semi-mature DCs, characterized by upregulation of important antioxidant enzymes i.e. SOD1, GSTO1, TXN, GCLM1 and regulatory proteins such as Prohibitin. These proteins all belong to the pivotal, cytoprotective Nrf2-Keap1 pathway, which is a major pathway in maintaining redox homeostasis. We believe that altered redox homeostasis, induced by continuous interaction with pathobionts, can be a crucial cofactor for developing intestinal inflammation and other autoimmune disorders. We postulate that symbiotic bacteria are able to trigger the activation of
cytoprotective, antioxidant pathways such as Nrf2-Keap1 pathway, which may contribute to their protective effect in IBD. All aforementioned proteins are summarily visualized in this study and can serve as a proteomics map to guide researchers for a better understanding of DC maturation and inflammatory conditions. Our proteomics catalogue also includes potential drug targets (e.g. Prohibitin, SOD1), which may be used for developing successful therapeutic strategies for IBD. Considering the immune regulatory properties of B. vulgatus mpk, shown by previous studies and this proteome study, the use of B. vulgatus mpk in probiotic combinations for treating intestinal inflammation may prove beneficial. We hope that our results will open new venues in the knowledge of dendritic cell maturation and associated mechanisms of intestinal inflammation.
Zusammenfassung

6 Zusammenfassung

Ziel dieser Arbeit war es, einen Einblick in die Rolle symbiotischer oder pathobiotischer kommensaler Bakterien bei der Pathogenese chronisch entzündlicher Darmerkrankungen zu bekommen. Mithilfe von Proteomics wurde das Proteinexpressionsprofil dendritischer Zellen untersucht, die entweder mit dem symbiotischen Stamm *B. vulgatus* mpk oder pathobiotischen Stamm *E. coli* mpk2 stimuliert wurden. Die Stimulation mit symbiotischen Bakterien führte zu

(i) einer verringerten Aktivierung und Maturation dendritischer Zellen,
(ii) einer reduzierten Inflammation und Migration dendritischer Zellen
(iii) einer verringerten Akkumulation reaktiver Sauerstoffspezies (ROS)
(iv) einer Aktivierung von Gegenregulationsmechanismen gegen oxidativen Stress.

Die Fähigkeit von *B. vulgatus* mpk, dendritische Zellen in semimature dendritische Zellen zu differenzieren und
somit die komplette Maturation zu verhindern, lässt sich an z. B. der Hemmung der proinflammatorischen Zytokinsekretion (TNFα, IL-6, IL-12p40 und IL-1β), sowie unterschiedlichen Expressionsmustern von aktivierungs- und maturationsassoziierten Oberflächenmarkern wie z.B. MHC Klasse II und CD40 erkennen. *B. vulgatus* mpk stimulierte dendritische Zellen zeigten zudem eine Herunterregulation proinflammatorischer Signalwege und Prozesse, indem z. B. Proteine, die zum TLR4 Signalweg, zur Inflammasom-Bildung, sowie zur Migration bei Entzündungsprozessen gehören, in geringerem Maß, als bei maturen dendritischen Zellen, exprimiert wurden. Ergänzend konnten wir zeigen, dass antiinflammatorische Proteine, wie z.B. TGFβ, in Folge einer *B. vulgatus* mpk Stimulation hochreguliert wurden, was auf eine Beteiligung semimaturer dendritischer Zellen bei der Entstehung von peripherer Toleranz hindeuten könnte. Darüber hinaus konnten wir zeigen, dass dendritische Zellen mit einer geringeren Menge ROS auf eine Stimulation mit *B. vulgatus* mpk reagierten als auf eine Stimulation mit *E. coli* mpk2. Zusätzlich konnten wir eine Steigerung des antioxidativen
Potentials semimaturer dendritischer Zellen nachweisen, das durch die Hochregulation wichtiger antioxidativer Enzyme wie SOD1, GSTO1, TXN, GCLM1, sowie regulatorischer Proteine wie beispielsweise Prohibitin gekennzeichnet war. Diese Proteine gehören zum Nrf2-Keap1 Signalweg, der eine große Bedeutung bei antioxidativen und zytoprotektiven Prozessen hat und damit entscheidend für die Aufrechterhaltung der Redoxhomöostase ist. Wir denken, dass eine veränderte Redoxhomöostase, die durch eine kontinuierliche Exposition gegenüber Pathobionten hervorgerufen wird, ein entscheidender Kofaktor für die Entstehung chronisch entzündlicher Darmerkrankungen und anderer Autoimmunerkrankungen ist. Wir postulieren, dass symbiotische Bakterien in der Lage sind zytoprotektiv und antioxidativ zu wirken, indem sie Signalwege wie Nrf2-Keap1 aktivieren und somit einen Schutz vor chronisch entzündlichen Darmerkrankungen vermitteln können. Die oben genannten Proteine sind ein Teil der graphisch dargestellten, in dieser Studie identifizierten, differentiell regulierten Proteine. Diese “Proteomics map” kann anderen Wissenschaftlern, beispielsweise im Rahmen von
Zusammenfassung

7 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>·O$_2^-$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>·OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>$^1$O$_2$</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Actn4</td>
<td>Actin, alpha 4</td>
</tr>
<tr>
<td>Aif</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>AKT1</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>AREs</td>
<td>Antioxidant response elements</td>
</tr>
<tr>
<td>Arg1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>Bacteroides vulgatus</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bradford standard assay</td>
</tr>
<tr>
<td>BV</td>
<td>B. vulgatus</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>CCAAT-enhancer-binding protein β</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>Casp1</td>
<td>Caspase 1</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CCL5</td>
<td>Chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Coro1</td>
<td>Coronin-1a</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosin-Phosphat-Guanin</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DCF</td>
<td>Dichlorofluorescein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2,7-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (for exmple)</td>
</tr>
<tr>
<td>EC</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fn1</td>
<td>Fibronectin 1</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box protein 3</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut associated lymphatic tissue</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td>GCL</td>
<td>Glutamate-cysteine ligase</td>
</tr>
<tr>
<td>GCLM</td>
<td>Glutamate-cysteine ligase, modifier subunit</td>
</tr>
<tr>
<td>GF</td>
<td>Germ free</td>
</tr>
<tr>
<td>Glns</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GRx</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>GSTO1</td>
<td>Glutathione s-transferase omega-1</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat-shock protein</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est (that is)</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>Ifi75</td>
<td>Interferon-induced protein 75</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-binding protein</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>mAB</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Mcl1</td>
<td>Induced myeloid leukemia cell differentiation protein</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph nodes</td>
</tr>
<tr>
<td>Mn</td>
<td>Manganese</td>
</tr>
<tr>
<td>Mo</td>
<td>Mock</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MPs</td>
<td>Mononuclear phagocytes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>Myh9</td>
<td>Myosin, heavy chain 9</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NDRG1</td>
<td>N-Myc Downstream Regulated 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Description</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>p62/SQSTM1</td>
<td>Ubiquitin binding protein 62/Sequestosome-1</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PHB</td>
<td>Prohibitin</td>
</tr>
<tr>
<td>PPRs</td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td>PSC</td>
<td>Primary sclerosing cholangitis</td>
</tr>
</tbody>
</table>
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>QBiC</td>
<td>Quantitative Biology Center</td>
</tr>
<tr>
<td>RO-</td>
<td>Alkoxy radical</td>
</tr>
<tr>
<td>ROO-</td>
<td>Peroxy radical</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RS-</td>
<td>Thiol radical</td>
</tr>
<tr>
<td>Rtn4</td>
<td>Reticulon-4</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Ship1</td>
<td>SH2 domain-containing inositol 5'-phosphatase 1</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Stat</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>Strep.</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T-cell</td>
</tr>
<tr>
<td>Tris</td>
<td>Trisaminomethane</td>
</tr>
<tr>
<td>TXN</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
</tbody>
</table>
8 References


References


Crohn's disease: role of CARD15 3020insC mutation?  
*Gut, 55*(3), 342-347. doi:10.1136/gut.2005.065557


Gee, K., Guzzo, C., Che Mat, N. F., Ma, W., & Kumar, A. (2009). The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. *Inflamm Allergy Drug Targets, 8*(1), 40-52.


Angiogenesis, 17(2), 335-345. doi:10.1007/s10456-013-9407-0


Khor, T. O., Huang, M. T., Kwon, K. H., Chan, J. Y., Reddy, B. S., & Kong, A. N. (2006). Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-


References


J Gastroenterol, 17(33), 3761-3775. doi:10.3748/wjg.v17.i33.3761


References


9 Contributions

Prof. Dr. med. Julia-Stefanie Frick designed the project, obtained funding for research and proofread the manuscript.

T. Popov performed the experiments illustrated in Fig. 6, 7, 11, 12, 13 and collected the data for Fig. 10 and 14. Under the supervision of A.G. Korkmaz, he performed the laboratory work (ELISA, FACS Western blot and ROS assay), data collection and analysis of the proteomics experiment, illustrated in Fig. 8, 9. He was involved in the concept planning of the study and wrote single-handed the manuscript.

A.G. Korkmaz was involved in the concept planning of the study, data collection and data analysis. He provided day to day supervision and proofread the manuscript of the thesis.
Prof. Dr. Boris Maček and Dr. Ana Velic performed the mass spectrometry.

Dr. Marius Codrea (QBiC center, Tübingen) performed the raw mass spectrometry data analysis and the statistics for the proteome experiment, illustrated in Fig. 8, 9.

Dr. Jörg Bernhardt compiled the Voronoi treemaps (Fig. 9) for visualization of the proteomics results.

DFG funded the research.

T. Popov was funded by SFB 685 with a MD stipend.
10 Acknowledgement

I want to sincerely thank Prof. Dr. Julia-Stefanie Frick for giving me the opportunity to work on this interesting topic and for the excellent supervision and support during my doctoral thesis. I am grateful for the constructive tips she gave me and for doing the proofreading at the end.

I want to thank Prof. Dr. I. B. Autenrieth for the possibility to execute my doctoral thesis in his institute and for the perfect working conditions I was provided with.

I owe a debt gratitude to my supervising tutor, Ali Giray Korkmaz, for teaching me how to perform microbiologic experimental methods, for patiently answering all my questions, for helping me out in the lab, for guiding me throughout the writing of my thesis with constructive tips and for doing the proofreading.
I want to thank all co-authors Prof. Dr. Boris Maček, Dr. Ana Velic, Dr. Marius Codrea and Dr. Jörg Bernhardt for their contributions.

I express my gratitude to the whole “AG Frick” for the fast integration in the group, for the professional help in the lab, for the nice working atmosphere and for the wonderful time we spent together.

Finally, I want to thank my family and friends, especially my sister Mariana Popova and my mother Maria Popova for motivating me during both experimental and writing phase of my doctoral thesis. I am grateful to my father Dr. Georgi Popov for professionally advising me and to P. Jourebanov for helping me to design some of the figures.
11 CV

Personal information

Name: Todor Popov
Date of birth: 03. Juni 1991
Place of birth: Sofia
Nationality: Bulgarian

Education

2010–2017 Eberhard-Karls University of Tübingen
30.05.2017 Completed final part of the medical licensing examination at the University of Tübingen
14.04.2016 Completed second part of the medical licensing examination at the University of Tübingen
<table>
<thead>
<tr>
<th>Date</th>
<th>Event/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.08.2012</td>
<td>Completed first part of the medical licensing examination at the University of Tübingen</td>
</tr>
<tr>
<td>2005–2010</td>
<td>91. German High School “Prof. K. Galabov”, German department, Sofia (Bulgaria)</td>
</tr>
<tr>
<td>2002–2005</td>
<td>Sofia High School of Mathematics „Paisii Hilendarski“, Sofia (Bulgaria)</td>
</tr>
<tr>
<td>1998–2002</td>
<td>Primary school „118. Liudmil Stoyanov“, Sofia (Bulgaria)</td>
</tr>
</tbody>
</table>

**Promotion**

<table>
<thead>
<tr>
<th>Date</th>
<th>Event/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014–2016</td>
<td>Institute of Microbiology, Tübingen</td>
</tr>
<tr>
<td>2014</td>
<td>SFB scholarship</td>
</tr>
</tbody>
</table>
04-06 Juli 2014 7th Seeon Conference on Microbiota, Probiota and Host" in Seeon, with a Poster: “Dendritic cells – a proteome approach”

23-25 Juni 2014 Annual Retreat of the Immunology Training Network of Erlangen, Tübingen and Würzburg at Kloster Schöntal

27 - 30 April 2013 23. ECCMID (European Congress of Clinical Microbiology and Infectious Diseases) in Berlin with a poster: “Prevalence of viral hepatitis, HIV and syphilis among inmates of Bulgarian prisons”