Towards a better understanding of chicken macrophage function in interkingdom signaling with postbiotic SCFAs

von

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For my family, friends and the chickens, because they made the greatest sacrifice

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ABBREVIATIONS

16S rRNA	16S ribosomal acid
18S rRNA	18S ribosomal RNA (represents Svedberg units)
28S rRNA	28S ribosomal RNA (represents Svedberg units)
APC	Allophycocyanin
APC-Cy7	Allophycocyanin- Cyanine 7
AvBDs	avian beta defensins
approx.	approximately
Aqua dest.	aqua destillata
BL buffer	lysis buffer
Ca^{++}	calcium ionized
C	carbogen
C2	abbreviation for acetate
C3	abbreviation for propionate
C4	abbreviation for butyrate
°C	degree Celsius
cDNA	coding deoxyribonucleic acid
CD4	cluster of differenciation 4
CD8	cluster of differenciation 8
CD40 ligand	cluster of differenciation 40 ligand
cGMP	cyclic guanosine monophosphate
$CH_3(CH_2)_2COOH$	empirical chemical formula for butyric acid
CH_3CH_2COOH	empirical chemical formula for propionic acid
CH_3COOH	empirical chemical formula for acetic acid
chCD45	chicken cluster of differentiation 45
chMQ	chicken macrophage
CHS	chicken serum
chTCR yδ	chicken T-cell receptor gamma delta
cm^2	square centimeter
CoA	coenzyme A
CO_2	carbon dioxide

CSF	colony stimulating factor
CT	cecal tonsil
ΔCT	delta cycle threshold
CV	conventionally housed
CX3CR1	chemokine (C-X-3C motif) receptor 1
CXCL8	chemokine (C-X-C motif) ligand 8
d	day
DAMPs	damage associated molecular patterns
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonucleic acid enzyme I
DTT	dithiotreitol
eNOS	endothelial nitric oxide synthase
ED	embryonic day
EDTA	Ethylenediaminetetraacetic acid
EtOH	ethanol
F71D7	isotype control
FBS	fetal bovine serum
FFAR	free fatty acid receptor
FITC	Fluorescein isothiocyanate
FoxP3	forkhead box P3 protein
g	gram
GALT	gut associated lymphoid tissue
GF	germ-free
GPR41	G-protein coupled receptor 41
GPR43	G-protein coupled receptor 43
GPR109A	G-protein coupled receptor 109A
GIT	gastrointestinal tract
H^+	hydron
h	hour
HBSS	Hanks' balanced salt solution
HDAC	histone deacetylase
HCO_3-	hydrogen carbonate

H_2O_2	empirical chemical formula for hydrogen peroxide
HOCl	empirical chemical formula for hypochlorous acid
IL1-ß	interleukin-1 beta
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
IL-8L1	CXCL8-like chemokine 1
IL-8L2	CXCL8-like chemokine 2
IL-10	interleukin-10
IL-13	interleukin-13
IL-18	interleukin-18
IL-34	interleukin-34
IEL	intra epithelial lymphocyte
I.U./ml	international unit per milliliter
IFN-ß	interferon beta
IFN-y	interferon gamma
Ig	immunoglobulin
IgA	immunoglobulin A
IgG1	immunoglobulin G1
IgG2a	immunoglobulin G2a
IgM	immunoglobulin M
IgY	immunoglobulin Y
iNKT	invariant natural killer cell
iNOS	inducible nitric oxide synthase
K^+	potassium ion
KUL01	monoclonal antibody (detecting MRC1L-B)
L	liter
LPS	lipopolysaccharide
M1	classification of macrophage type 1
M2	classification of macrophage type 2
M	molar mass
mAb	monoclonal antibody

MCT type 1	monocarboxylate transporter type 1
Mg^{++}	magnesium ion
mg	milligram(s)
MHC class I	major histocompatibility complex class I
MHC class II	major histocompatibility complex class II
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mM/L	millimoles per liter
$MnCl_2$	manganese-(II) chloride
MPS	mononuclear phagocytic system
MRC1L-B	mannose receptor C-type 1L-B
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response protein 88
Na^+	sodium ion
NADPH	(reduced) nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B-cells
ng	nanogram
NK	natural killer cell
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2-	nitrite
NO_3-	nitrate
O_2-	superoxide
·OH	hydroxyl radical
Pam2CSK	Pam2CysteinSerineLysine4
PBS	phosphate-buffered-saline
PRRs	pattern recognition receptors
pH	potential of hydrogen (logarithmic)
pKa	negative base-10 logarithm of the acid dissociation constant (Ka)
qPCR	quantitative polymerase chain reaction

RIN	RNA integrity number
RNA	ribosomal ribonucleic acid
RNA-Seq	RNA sequencing
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
RPL-13	ribosomal protein L13
RT	room temperature
s	second
SCFA	short chain fatty acid
SLC5A8/12	sodium-coupled monocarboxylate transporter 5A8/12
SMCT	sodium-coupled monocarboxylate transporter
TCR1	T-cell receptor 1
TCR-ß	T-cell receptor beta
TGF-ß	transforming growth factor beta
TG buffer	thioglycerol buffer
Th1	type 1 T helper cell
Th2	type 2 T helper cell
Th17	T helper cell 17
TIR	Toll-interleukin 1 receptor
TNF-α	tumor necrosis factor alpha
TLR2	toll like receptor 2
TLR4	toll like receptor 4
TLR15	toll like receptor 15
TRAM	translocating chain-associated membrane protein
Tregs	regulatory T-cells
xg	times gravity (in terms of centrifugation)
µl	microliter
µm	micrometer

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1 INTRODUCTION

“All disease begins in the gut” – Hippocrates, the father of modern medicine, is said to have made this statement more than 2000 years ago. Regardless of its unconfirmed authenticity the inherent wisdom influenced many researchers over the last centuries up to modern times (1) including Leuvenhoek, the father of protozoology and bacteriology and the nobel prize winner Metchnikoff, the father of innate immunity and discoverer of the significance of phagocytosis in development, homeostasis and disease, who were inspired by the coexistence of the host and the associated microbiota as well as by the mutual regulation of microbe populations (2).

Mammals, as well as chickens and their associated immune system have evolved over millions of years in the context of microbial colonization, resulting in a mutually dependent inter-kingdom and transkingdom (especially viruses) networking relationship and therefore coining the concept of a meta-organism, permanently associated with at least as many microbial cells as somatic cells, potentially comprising about 100 times more genes within just the gut microbiota than the host itself (1, 3-16). Commensal, pathogenic or symbiotic relationships between these organisms and the host cells are known (14, 17) and the evolution of vertebrates is also associated with longer life and so a higher number of symbiont generations per host generation (18). Based on this characterization, the holobiont hypothesis was first introduced by Lynn Margulis in 1991, initially referring to a simple biological entity involving a host and a single symbiont, now it comprises- a host (animal or plant) and its associated microorganisms living in or on it- therefore also challenging the original definition of immunology as “science of self non-self discrimination” (11, 18-21).

The interrelationships of this finely balanced coexistence have been and are currently being investigated in various experimental approaches.

In 2008 the “human microbiome project” was initiated, aiming to firstly create a reference microbiome of healthy individuals of different sampling sites: nasal and oral cavities, skin, gastrointestinal and urogenital tract, secondly, to explore the possible impacts of the microbiome composition on particular human diseases and thirdly, to focus on the postbiotic properties of the microbiome such as metabolites, proteins and gene expression profiles under certain conditions such as diabetic patients or pre term birth. Based on results of this project diagnostic, therapeutical as well as predictive approaches such as sequence based analyses of donor and recipient stool are now made to treat microbe associated illnesses (22, 23). This project will certainly give insights into the host to microbe, microbe to microbe and microbe to host controlling signals in health and disease, as a constantly growing field of “gut-reference

organ" axes and microbial associated systemic diseases shapes the research landscape in mammals as well as in chickens (1, 24-32).

Another growing field of interest examines microbiota present in areas of the mammalian or avian body originally considered sterile such as blood, bones, breasts and amniotic fluid (24, 33-40). Some diseases are now associated not only with gut dysbiosis but with tissue dysbiosis. These include, for example, diabetes, asthma, obesity in humans as well as chondronecrosis with osteomyelitis in chickens (24). Using 16S rRNA sequencing methods Gong was able to detect microbiota derived from the oviduct of the hen in the chicken embryonic gut (41). Moreover, Salmonella bacteria are often present in the chicken blood without causing clinical symptoms (42). Although using 16S rRNA methods are controversially discussed (43, 44), ultrastructural microscopic methods seem to verify the presence of microorganisms in "germ-free" compartments (45).

Models with germ-free animals demonstrate that some disease phenotypes, for example adiposity, metabolic syndrome, colitis or epilepsy can be transmitted with the ingesta into germ-free healthy recipients (46-48). In contrast, microbiota in form of fecal microbial transplants can be protective against diseases, for instance against the onset of type 1 diabetes in mice or the infection with Salmonella enteritidis in newly hatched chickens (49-51). Early and current studies with germ-free housed chickens displayed a profoundly underdeveloped local and systemic immune system (52-56). Transplantations of microbes from healthy donors to other individuals is already a common practice in livestock (57). In addition, synthetic microbial consortia, adult-derived microbiota, prebiotics, probiotics or bioactive microbial compounds have been found to effectively boost the immune system or help to displace harmful microbes when administered to chickens in ovo, early after hatching, after a change in diet or in case of dysbiosis (17, 58-60).

Not least because of the corona crisis, researchers are currently also looking into the interactions between the host microbiota and vaccination as well as on individual drug metabolism alterations due to the composition of the microbiota in mammals (61) as well as in chickens (14, 62-70). Moreover, in vertebrates bacterially derived metabolites or vesicles reach immunologically privileged areas as well as peripheral organs and serve as means of communication with host (immune-) cells and even might influence behavior (1, 71-76).

In 2022, Diener et al. found that about 64% of 930 investigated blood metabolites in the human species are associated with either host genetics or gut microbiome. 69% of these molecules solely refer to gut bacterial origin, only 15% were solely driven by genetics and 16% were of joint control of both genes and the microbiome (77). The results of this study imply that the bacterial composition and thus also the bacterially produced metabolites with all their peripheral physiological aspects can be influenced by diet. In the chicken, holistic knowledge

about the influence of gut microbiota on serum metabolite levels is quite scarce. Therefore, nutritional immunomodulation is an interesting upcoming field of research in the mammalian as well as in the avian world and works through targeting the ecology and composition of the microbiota, intending to prevent or ameliorate the outcome of health issues (60, 78-84).

Those bacterial derived metabolites include for example short chain fatty acids (SCFAs) such as butyrate, propionate and acetate besides essential vitamins and amino acids amongst many others (72). Especially butyrate has gained much attention because of its mainly beneficial effects as a feed supplement in poultry for example on weight gain or resistance and antimicrobial effects to enteric pathogens (84-89).

In the interaction with bacteria, macrophages play a crucial role. They function as sentinel cells of the innate immunity and through their pattern recognition receptors they recognize for example invading bacteria (16). They play a critical role in homeostasis, development and during the onset and control of immunological responses and therefore also in regulating of T- and B-cell functions trough the production of cytokines (90). In mammals, the largest accumulation of macrophages is located in the lamina propria underneath the gut epithelium (91). There they also sustain and expand the number of FoxP3 positive regulatory T-cells (Tregs) which are essential for maintenance of gut integrity (92).

However, while numerous studies in mammals examine the anti-inflammatory influence of SCFAs, particularly butyrate, on physiological functions and on many cell types of the myeloid and lymphoid cell lineage (1, 93-117), there are only few studies in chickens that examine the immune regulatory effects on chicken monocytes (118), bone marrow derived cells (86), whole homogenized organ segments of duodenum, jejunum, cecal tonsil and crop explants (86) and chicken macrophage cell lines such as HD11 (86, 119-122) and HTC (27, 90, 118, 121, 123, 124).

One of the central questions is why fulminant inflammatory responses do not constantly occur in the gut or systemically as bacteria resp. bacterial products translocate in mammals (125, 126) as well as in avians (127, 128). Given that the intestine hosts the largest concentration of resident immune cells in both human and avian bodies (16, 129, 130), the immune system, especially within this organ, somehow has to balance tolerance to commensal bacteria and be able to respond adequately to potential pathogens, sharing similar molecular patterns (131).

This dissertation presents first basic insights towards a better understanding of the effects of SCFAs, mainly butyrate on the chicken macrophage cell line HD11 as well as on primary cells derived from blood, bone marrow, spleen, ileum and cecum.

2 LITERATURE REVIEW

2.1 Microbial modulation of the chicken immune system- an overview

A common mucosal system protects the body from external pathogens. Influencing the microbiota of the gastrointestinal tract (GIT) tract results in alterations of other lymphatic mucosal systems and primary and secondary lymphatic or systemic organs in various experimental approaches in mammals as well as in avian species (30, 132). Homing processes lead to the linkage of different mucosal areas by migration of stimulated immune cells through other mucosal sites and there is also considerable traffic of immune cells between different gut immune structures and systemic organs such as the spleen and the bone marrow (133, 132).

On the other hand, viable bacteria from the intestinal lumen translocate to gut distal sites in both mammals (125, 126, 134) and chickens (127, 128). Some gut derived bacteria are believed to use the “Trojan horse” strategy to survive for example in macrophages and use those cells for systemic dissemination (16, 135, 136). Moreover, blood and tissue dysbiosis is an upcoming field of research in mammalian as well as in avian species (24).

2.1.1 Impact of microbial colonization pre hatch

Development of the chicken immune system prior to hatch has been thought to be antigen-independent. Nevertheless, recent work suggests differently:

In 2020, Akinyemi et al. investigated the chicken embryonic gut microbiota at different developmental stages (embryonic day (ED) 3, 12 and 19) by using 16S rRNA methods and found 21 phyla, 601 genera with 96 genera resembling core microbiota at those three stages of development (137).

During and after oviposition, the cuticula, egg shell and underlying membranes can be penetrated by several bacteria as reviewed by Gantois et al. (138).

If not due to contaminations, these findings suggest a possible early encounter of the embryo and its developing immune system with microbiota.

Recent work from Gong et al. demonstrated, that during the formation of the egg in the maternal reproductive tract, immune factors and maternal microbiota of the oviduct, both dependent on the maternal immune system, are transferred into eggs subsequently influencing the morphology of the embryonic gastrointestinal tract besides increasing the levels of immunoglobulines (Ig) IgA, IgY, IgM and avian beta defensins (AvBDs) in egg white and the

levels of IgA, IgY, and IgM in yolk (41). Based on the results of Ding et al. (139), who correlated the embryonic microbiota with the maternal fecal microbiota, and Lee et al. (140), who suggested the seeding of the egg white with maternal bacteria from the oviduct, Gong also used a 16S rRNA approach and found that large proportions of microbes in the egg white and embryonic gut were sourced from the maternal magnum during egg formation.

Besides vertical transmission, artificial manipulations at pre-hatch state are common practice and might also give insights into the potential interplay of microbiota (or microbial derived antigens) and the capacity of the embryonic immune system. Some examples are listed below.

In ovo immunization with several vaccination forms (live attenuated, subunit, adenovirus-vector based, recombinant protein vaccines) is an established method to protect chickens against several pathogens including Marek´s disease, Inflammatory bowel disease, fowl pox, Newcastle disease and coccidiosis (16, 141). Administration of recombinant Salmonella flagellin into the amniotic sac results in an intestinal cytokine response (interleukine 6 (IL-6) and IL-8) as well as toll like receptor 15 (TLR15) expression but not in the spleen 24h post vaccination. Moreover, IgY antibodies in the serum were induced and could be detected up to 21 days post hatch (142).

Duan et al. found, that in ovo injection of probiotics (Lactobacillus plantarum) and synbiotics (Astragalus polysaccharide and Lactobacillus plantarum) at 18.5 days of incubation significantly increased not only the feed intake, body weight, and the feed conversion ratio after hatching, but especially the administration of synbiotics enhanced the levels of serum IL-2, interferon-y (IFN-y), and secretory immunoglobulin A in intestinal lavage fluid and the histomorphological development of the small intestine. Furthermore, synbiotic injection significantly increased Lactobacillus and Bifidobacterium colonization while decreasing the relative abundance of Escherichia coli in the chicken cecum (58).

Also implementing an in ovo strategy, Pender showed that a commercial probiotic product (Primalac) resulted in an initial upregulation of inducible nitric oxide synthase iNOS at hatch and a downregulation of TLR2, TLR4, iNOS, trefoil factor 2, IFN-y, IL-4 and IL-13 in the ileum and the cecal tonsil of broilers (143).

The in ovo administration of synbiotics resulted in enhanced colonization of the GALT (gut associated lymphoid tissue) of ROSS 308 broilers by B- and T-cells at day (d) 7 and 21 post hatch besides other impacts on primary and secondary lymphoid organs (144).

Using an in ovo approach, Alizadeh et al. evaluated the effect of early colonization of the chicken intestine with lactobacilli on the development of immune competence in newly hatched chicks. The expression of several genes such as IFN-ß, IFN-y, transforming growth factor beta (TGF-ß) and IL-18 was downregulated in the spleen after in ovo administration of lactobacilli.

This effect was absent in the bursa of Fabricius. Moreover, the in ovo administration of 10^7 colony forming units of lactobacilli increased the number of KUL01+ cells in the spleen on day 5 post-hatch, while no significant difference was observed in chickens that received lactobacilli through both in ovo and oral routes (145).

Further studies are required in this field to investigate the relevance of these findings in the context of the development of the embryonic immune system.

2.1.2 Impact of microbial colonization after hatch

After hatch, experiments involving feed, antibiotics, hygiene management as well as studies with germ-free or gnotobiotic animals provide insights on the influence of the gut microbiota on immune system development:

Bar-Shira et al. found that delayed access to feed and water, a common problem in chicken industry due to variations in hatching time, caused significantly delayed GALT activity especially in the hindgut (cecum and colon). Colonization of the cecum and colon by T- and B-cells was delayed and systemic and intestinal antibody responses following rectal immunization were lower. This effect lasted for about two weeks. Interestingly, the development of the foregut (duodenum, jejunum, ileum) was only slightly impaired (146).

Engberg et al. showed that innate factors such as pancreatic enzyme activity and pH are influenced by feed grinding and feed form. Moreover, in this study the composition of the gut microbiota and the fermentation of SCFAs were also differently affected (147). Furthermore, feeding raw and variously processed peas resulted in a quantitative increase in intraepithelial T-cells in the jejunum of broilers, suggesting an immunomodulatory effect of peas (148).

Schokker et al. examined the short-term (24h) effect of orally administered amoxicillin in 1-day old chicks. Besides changes in the composition and diversity of the microbiome and in the expression of numerous genes, immunohistochemistry of the jejunum displayed a reduced number of KUL01+-macrophage-like cells due to direct and indirect effects of the amoxicillin treatment. Therefore, whilst gut innate immunity is well developed at hatch, the initial colonizing microbiota can influence immune features days or even weeks later (149).

Comparing two models of litter hygiene Butler et al. recognized a reduction in defensine expression (AvBD 1 and 4) at hatch and at day 7 post hatch in the duodenum and cecum contributing to a high hygiene management (150).

Early and current studies with germ-free housed chickens displayed a profoundly underdeveloped local and systemic immune system (52, 54, 56, 151). Hedge et al. recognized

a significant reduction in cecal tonsil lymphoid tissue, including complete absence of lymphoid follicles, whilst there were no consistent differences in immune organ (bursa, thymus or spleen) weights. Although based on inconsistent results Hedge also recognized a reduced number of plasma and lymphoid cells in thymus, spleen and bursa in germ-free (GF) quails (56).

Honjo et al. investigated the distribution of lymphocytes in lymphoid organs in GF animals compared to conventionally housed (CV) chickens on an immunohistochemical level and also found poorly developed lymphoid follicles in the cecal tonsils, lacking IgY and IgA positive cells, a reduced number of B- and T-cells in the villous region of the cecal tonsils, whereas similar distribution of B-cells in the spleen, bursa and thymus was found. In addition T-cells were distributed more widely in the spleen in CV animals than in GF animals (54).

Cheled-Shoval et al. showed that GF chickens have lower neutral and acidic goblet cell number and density, and mucine 2 expression was reduced in the ileum of GF and mono-associated animals, although the germinal center density increased in the cecum of GF and monoassociated chickens (similar observations were made in gnotobiotic pigs and rodents) (152).

Mwangi et al. demonstrated the dependencies of microbial complexity on the αβ T-cell receptor repertoire in the chicken gut by comparing GF, mono-colonized and conventional chickens (153).

Using a high throughput sequencing method Dascalu et al. showed that the microbiome of the intestinal and the bursal site is an important driver of T-cell receptor beta (TCR-ß) diversity. This effect was absent in splenic tissue (52).

Lettmann et al. showed the absence of CD4+ and CD8+ T-cells in gut tissues of GF animals. Moreover, B-cells were missing in the lamina propria. In addition, not until day 28 post hatch, germinal centers were absent in the cecal tonsils and no intestinal IgA or serum IgA was found. These findings were partly reversed by administration of a mixture of four different bacteria or by mono-association by an E. coli strain. GF chickens had a non-selected polyclonal population of T-cells and mono colonized chickens displayed a biased repertoire in the gut at 21 days of age compared to conventional housed chickens. Macrophages and heterophils were present at hatch and fully functional to produce cytokines and chemokines when challenged with Salmonella enterica (55).

Zenner et al. showed that the early application of maternal microbiota to the chick causes increased IgA and IgY levels in the cecal tonsil and a synthetic microbial consortium increased serum leves of IgA and IgY (17). In a similar approach, Volf et al. detected differences in gene expression profiles in chicken cecal tissue (154).

Another factor shaping the immune system and the microbial composition after hatch are infections with pathogens.

Pathogen related local inflammation induces enterocyte differentiation and proliferation in the crypt to replace damaged enterocytes at the villus tip (130) and tight junction formation can be altered by pathogenic microbes, virulence factors, as well as commensals (14).

Artificial modulation of the microbiota, mainly the gut microbiota in form of prebiotics (155) and probiotics in mammals as well as in chickens, with the purpose of (nutritional) immunomodulation is commonly used already (80) and was also found to modulate immune responses to vaccinations. These effects are also linked to SCFAs production (65, 66, 79, 156).

The effect of the intestinal microbiota on the response to vaccination is subject of recent research. In humans, different responses to oral vaccines seem to be related to different gut microbiomes. Effects influencing the composition of the microbiome such as diet or hygiene might therefore impact the immune response to vaccination. Moreover, it has been reported in both humans and chickens that the immune response to vaccines can be enhanced by orally administered probiotics (14).

Lee and Lillehoj were able to show that antimicrobial treatment affects the humoral and cellular immune response to vaccines in chickens (predominantly increase) (157).

Lyimu et al. showed that oral administration of Salmonella vaccines alters microbiota composition in the chicken ceca and gene expression profiles in cecal tissues (158).

One of the most important pathogens with zoonotic potential in the poultry industry are Salmonella spp.. Kogut et al. summarized the three stages of alterations in the chicken cecum after Salmonella infection. Salmonella seem to have evolved a unique survival strategy that minimizes host defenses during the initial infection following a dramatic immunometabolic reprogramming in the cecum that alters the host defense to disease tolerance resulting in a long-term persistent infection in the cecum. Moreover, it is well known that Salmonella survives in macrophages and uses those cells to shuttle to other sites in the chicken body (159).

On the other hand, the development of enteric resistance to Salmonella enterica (enteritidis and typhimurium) is also age dependent. Chicks less than 3 days of age are severely affected whilst infection of older chickens is largely restricted to the gut (159).

Studies with enteroids will certainly allow more insights into the immunological functions of enterocytes and leukocytes in the gut in general in the chicken (160, 161).

In summary, the microbiota seems to harbor the potential to exert local and systemic immunologic effects. Therefore, alterations of the microbial composition are most likely to influence the immune system vice versa.

There are different communication routes used by bacteria to interact with host cells and one of them is the production of metabolites, such as SCFAs.

2.2 Short chain fatty acids

The microbiota trains, stimulates and functionally adjusts the immune cells. The microbiota communicates with the host in different ways. One possibility is the production of metabolites. Short-chain fatty acids have been intensively studied across species in recent years. Among other things, because of their antibacterial and immune-modulating properties.

2.2.1 Definition

Short chain fatty acids (SCFAs) are normally defined as monocarboxylic acids with a hydrocarbon chain lengtht of 1 to 6 atoms (162). Other definitions include for example only molecules with chain length from 1 to 4 C atoms (163). This group comprises formic acid, acetic acid, propionic acid, butyric acid, valeric acid and caproic acid. In this work, we focus mainly on butyrate ($CH_3(CH_2)_2COOH$), but also investigate the role of propionate (CH_3CH_2COOH) and acetate (CH_3COOH) (162).

2.2.2 Physicochemical properties

In general SCFAs are amphipatic molecules (164, 165) consisting of a hydrophobic hydrocarbon chain on one end and a hydrophilic carboxylic tail on the other and their lipophilicity (166) increases as the number of carbon atoms increases. Butyrate, propionate and acetate are week acids with a pKa of 4.75 for acetate, 4.88 for propionate and 4.81 in the case of butyrate (162). At pH 7 (25°C) the anionic form is predominant. The inner body temperature of chickens is about 40°C, which means that the pKa of those SCFAs might be lower and therefore the anionic form might also be more predominant in the chicken body. In the chicken gut, the pH values vary locally due to for example bile acid secretion the pH is higher in the ileum, whereas in the cecum the pH is lower due to fermentational processes but still higher than the pKa value of acetate at room temperature (167) which means that the SCFAs might also be present in the anionic form. At the site of inflammation lower pH values are present and might also interfere with the anionic form (168). Interestingly, incorporation of SCFAs into bilayer membranes is known to increase their pKa (162). SCFAs permeate the inner mitochondrial membrane in the non-esterified form and are used as substrates in the mitochondrial ß-oxidation (162). Comprehensive studies considering the local and systemic properties and different circumstances in the chicken body are lacking. The odor of those SCFAs can be described as sharp or rancid (162). Binding to cations ($Na^+/K^+/Mg^{++}/Ca^{++}$) leads to the formation of salts and reduces odor (169). Onrust at al. investigates different

formulations of butyrate used in poultry display different availabilities and sequalae in the GIT, especially in the cecum (170).

In this study, only sodium salts dissolved in the cell culture medium at the desired concentration were used.

2.2.3 Production

The process of bacterial SCFAs production is seen as sign of coevolution of host and microbe because SCFAs are produced as metabolic waste products and the host is unable to produce these important metabolites by itself (171). Kaiko et al. presented evidence that the crypt morphology in humans and higher order mammals may has evolved to protect intestinal stem cells from the antiproliferative effects of butyrate (100). Nevertheless, butyrate is also produced outside the host (171) and also appears in some plant oils and milk (butyrate, acetate) bound to triglycerides (172-174) as well as in the sweat, genital tract and oral cavity (162, 169, 175, 176).

The main site of SCFAs production is the gastro intestinal tract (30). Undigested food components reach the large intestine and are available for microbial metabolism and energy production. These include residues of fats and proteins, mono- and disaccharides and resistant starch in addition to non-food components such as endogenous secretions, digestive enzymes, mucus and exfoliated epithelial cells (163). Mainly undigestable fiber and resistant starch is used for bacterial production of SCFAs (72, 177). Jadhav et al. provides an overview of selected studies on SCFA producing bacteria in the chicken (177). Rehman et al. reviewed the SCFAs concentrations in the chicken ingesta under different conditions (feeding, age) and the composition of the fermenting bacteria at different sites of the avian body (167).

In the chicken, the main site of anaerobic fermentation is the cecum but SCFA producing bacteria are also found in the crop as well as in the ileum and colon (72, 167, 178-181). Additional endogenous production of free acetate is described in the liver and kidneys of mammals (182). Under ketogenic conditions acetate and ß-hydroxybutyrate are mostly produced by the liver (183). In the chicken, acetate can also be found in the lungs of germ-free animals, suggesting an endogenous production in this species (30, 184).

The main way of acetate production is the reductive methylation of CO_2 (Wood-Ljungdahl pathway) (162). Another way is the acetyl-CoA pathway (185). Propionate is produced through two main routes: the methylmalonyl-CoA pathway, where it is generated from lactate. By taking up lactate, propionic bacteria produce pyruvate which becomes carboxylated by

methylmalonyl-CoA-carboxyl transferase to oxaloacetate which is subsequently converted to propionate (through a four carbon pathway consisting of the intermediates malate, fumarate, succinate and methylmalonyl-CoA). This pathway also generates one acetate in addition to two propionate molecules. In the acrylate way the CoA ester of lactate (lactoyl-CoA) is converted via acryloyl-CoA to propionyl-CoA, which is then hydrolyzed to propionic acid.

The formation of butyrate requires two acetyl-CoA molecules which form one acetoacetyl-CoA molecule. Subsequently butyryl-CoA is formed through reductive conversion of acetoacetyl-CoA (162). In addition, butyrate can also be synthesized from proteins through the lysine pathway, which demonstrates that gut microbiota can accommodate changes in the fermentation substrate, with the aim of retaining metabolite synthesis (185). This process of bacterial cross feeding where acetate can be directly used by butyrate-producing bacteria has been shown to promote the growth of certain bacteria and therefore the diversity of microbiota in the gut which in turn may influence host metabolism (174). Another source of butyrate is the hydrolysis of milk lipids by salivary and gastric lipases (169). Besides the production by commensals through anaerobic fermentation, butyrate can also be produced by pathogens producing also harmful byproducts like ammonia (186). Branched chain fatty acids such as isobutyrate can also be found in the gut (187). Moreover, in form of ketone bodies ß-hydroxybutyrate and acetoacetate are also present in the chicken before and after hatching (188-190).

Liu et al. summarized the effects of supplementation of dietary fiber, prebiotics, probiotics and additives on SCFAs production in poultry. Probiotic supplementation of Lactobacillus ssp, Clostridium butyricum, Bacillus subtilis or multi-strain probiotics can significantly alter the SCFA ratio in young chicken. Feed additives in form of for example sodium butyrate, tributyrin and propionate have been shown to impact the production of SCFAs in poultry not only in the cecum but also in other parts of the chicken GIT such as the jejunum and ileum (185).

2.2.4 Absorption and transport mechanisms

In the chicken, there is limited information about absorption, transport processes and mechanism of action. Most studies refer to mammals. Earlier studies found that SCFAs are absorbed in the small and large intestine by similar mechanisms, whereas more recent studies suggest the existence of species differences and different transporter isoforms (162, 169). The non -ionic diffusion, SCFA/HCO_3- exchangers, Na-coupled monocarboxylate transporters (SMCT or SLC5A8/12) and monocarboxylate transporter type 1 (MCT type 1) transporters have been reported on the apical site of the gut epithelium in mammals (169, 191, 192).

On the basolateral site, a carrier-mediated, HCO_3- -gradient –dependent anion-butyrate exchange system is found. In sheep, anion competition experiments showed that SCFAs can be transported by bicarbonate dependent and independent mechanisms. In pigs and humans MCTs are involved in butyrate transport and this process seems to be pH dependent as the optimal pH of the colonic butyrate transporter seems to be 5.5. This transport is saturable, inhibited by several monocarboxylates such as acetate, propionate, pyruvate, L-lactate and a-ketobutyrate and dependent on H^+ (162, 169).

SCFAs are transported via the portal vein to the liver or via the vena cava caudalis and the lymphatic system into the systemic blood stream (193, 194).

The intestinal epithelium seems to consume most of the butyrate in mammals (116, 187). In mammals, SCFAs reach the superior mesenteric vein, inferior mesenteric vein and portal vein through passive diffusion and active transport by transporters mentioned above (C2, C3 and C4 concentrations are 262.8 mM/L, 30.3 mM/L, and 30.1 mM/L, respectively). Acetate and propionate are metabolized by the liver. The remainder of SCFAs reaches the peripheral circulation (the concentrations of C2, C3, and C4 were 172.9 mM/L, 3.6 mM/L, and 7.5 mM/L) (116).

SCFAs can also reach immunologically privileged organs such as the brain (108, 109), the eye (195, 196) or the placenta (197-199). Acetate, propionate and butyrate are detectable in the human cerebrospinal fluid and propionate and butyrate are also detectable in the brain tissue (108, 109). Moreover, the levels of butyrate in the brain tissue of mice supplemented with Clostridium butyricum exceeded plasma levels, therefore suggesting a possible accumulation process (108, 200).

Information on peripheral concentration levels in chickens is scarce. In the chicken, Saint-Martin et al. detected all three short chain fatty acids in the lungs of conventionally housed chickens, whereas only acetate could be measured in germ-free animals (30, 184).

2.2.5 Mechanisms of action

The same type of SCFAs can have different inhibitory or promoting effects on different types of cells, but different types of SCFAs also have different effects on the same types of cells (116).

SCFAs exert many effects on different cell types in mammals as well as in chickens. Current studies examine the effects on numerous host cells including fat cells, epithelial cells, fibroblasts in addition to involvement in neurological processes such as anxiety and memory

(72, 177). In addition, in mammals, many cell types of the myeloid and lymphoid immune cell line have been intensively studied for their interaction with SCFAs, including macrophages (73, 116), dendritic cells (93-95, 116), NK-cells (116), T-cells (116), B-cells (116), plasma cells (201), iNKTcells (96), ILCs (116), neutrophils (116), eosinophils and basophils (97, 116), mast cells (98, 99) as well as stem cells (100-102).

The mechanisms by which SCFAs affect cells of both the innate and adaptive branch of the immune system have been the subject of controversy and are yet to be thoroughly investigated. Liu et al. and Stein et al. reviewed information on G-protein coupled receptor (GPR41/GPR43/GPR109A) dependent and independent mechanisms, the interference with HDAC molecules and the inhibition of NF-kB through both GPR-binding and histone deacetylase (HDAC) inhibition as well as mTOR influencing pathways amongst several others (116, 202). In the chicken GPR43 (FFAR2) has more than 20 paralogs and the pharmacology of SCFA receptors and their mechanisms of action in the chicken are unresolved (30, 203). Only GPR43 has been identified on a molecular level in this species while there is literature suggesting the presence of GPR41 (FFAR3) (204) and GPR109A (32).

Nevertheless, by inhibiting monocarboxylate transporters in the absence of FFAR2 and FFAR3 (examined by single cell RNA-Seq) Caetano-Silva et al. suggested that SCFAs directly regulate microglia function following diffusion in mice (109).

Downstream effects on mammalian macrophages include the regulation of pro-inflammatory as well as anti-inflammatory cytokines, an altered immune cell metabolism and enhanced phagocytosis as reviewed by Liu et al. and Stein et al. (116, 202).

In the chicken information about the interaction of SCFAs on those cell types is comparatively scarce and includes Tregs (205), macrophage cell lines (86, 90, 119-122), monocytes (119, 120) and bone marrow derived cells (86). Lee examined the impacts of a SCFAs mixture (acetate, propionate and butyrate) in the context of an adenovirus infection. Under SCFAs treatment MHC class II expressing monocytes were enhanced, the numbers of T-cells and effector molecules in peripheral and lymphoid tissues were increased. Intraepithelial cells (IELs) produced more cytokines usually involved in pathogen elimination and changed the intestinal microbial composition (206).

2.3 Macrophages

In 1882, Ilya Ilyich Mechnikov, also known as Elie Metchnikoff, a Russian immunologist who is considered to be the father of phagocytosis, cellular innate immunity, probiotics and gerontology, described phagocytic cells in transparent starfish larvae inserted with tangerine tree thorns and later (1883/84) in Daphnia magna infected with fungal/yeast spores. He concluded that those cells provide defenses against non-specific infections (207-209). The process of phagocytosis per se was observed and interpreted earlier by numerous scientists before Metchnikoff. The earliest reports of phagocytic cells date from the mid-19th century, 300 years after the observations of Antony van Leeuvenhoek (207, 208). For example, in 1847, Alexander Ecker, an anthropologist described erythrocytes inside rabbit spleen cells. In 1871 Giulio Bizzozeo, an Italian professor of pathology provided the first pictures of macrophages that had ingested erythrocytes, a process now called efferocytosis. Bizzozeo also theorized that these cells might have important immunological functions defending pathogens (207). In contrast, in 1878 Robert Koch believed, that these cells contribute to the distribution of pathogens after observing numerous intracellular bacteria following artificial infections with anthrax (“Trojan horse theory”) (208). The main discovery of Metchnikoff was the understanding of this process. In his publication (1883) he described the role of phagocytes containing pieces of muscular fibers and nerves during the metamorphosis from the tadpole to frog and reported the phenomenon of phagocytosis in the absence of signs of inflammation (207). In an additional experiment a frog was injected with putrid blood and Metchnikoff observed that white blood cells particularly in the spleen contained variable amounts of bacteria. He also described phagocytosis in vertebrates (210). For this accomplishment, the Nobel Prize was presented to Elie Metchnikoff in 1908 (211).

Today, macrophages represent a heterogenous group in mammals as well as in chickens (16) including for example classical macrophages, brain microglial cells, liver Kupffer cells, epidermal Langerhans cells and bone osteoclasts (16, 212) as well as alveolar macrophages (213) amongst several others (212). Several subsets such as liver capsular macrophages and central vein macrophages are further distinguished in mammals (214). In the chicken, subclassifications can also be found for example in the spleen (160, 215) or in the gut (216). Another category is tumor-associated macrophages, which fight or promote cancer development and metastasis dependent on the cancer type and tumor environment (212).

Further transcriptomic analyses are needed to distinguish different macrophage subsets in the chicken.

The term “mononuclear phagocytic system” (MPS) replaced the term “reticuloendothelial system” (208) and includes all types of macrophages and their bone marrow progenitors (212).

2.3.1 Origin

Macrophages are ancient phagocytic immune cells that are thought to have originated 500 million years ago in metazoan phylogeny (217).

The prevailing view in the literature has been that tissue macrophages in health and disease originate from circulating monocytes. This has been challenged by evidence originating from congenic bone marrow transplantation in humans or genetic tracing strategies and cell tracking in the mouse (218).

Macrophages arise early in the embryonic phase (219). In the chicken, at ED2, cells with macrophage-like morphology have been found in the yolk sac with increasing numbers during development (220). In mammals, macrophages derive from hematological progenitor cells (from yolk sac, fetal liver, aorta-gonad-mesonephros and bone marrow hematopoiesis (16, 221, 222) along with dendritic cells, granulocytes, mast cells (223). The liver and spleen may also participate in the development of myeloid cells in mammals (224).

The precursors of chicken mononuclear phagocytic system cells still remain to be investigated. The development of chicken MPS cells is not exactly equivalent to the development of the mammalian MPS system and the exact origin of chicken specific MPS subsets including tissue macrophages is unknown. Thus, it is unclear whether mannose receptor C-type 1L-B (MRC1L-B) positive tissue resident macrophages derive from embryonic or hematopoietic stem cells. Interleukins such as IL-34 and members of the colony stimulating factor (CSF) are involved in the mammalian myelopoiesis and survival of myelogenic cells (222). CSF1 and IL-34 seem to be conserved in birds and vertebrates in general (225). However CSF1 has been shown to have no effect on blood monocytes in 1-week old chicks, suggesting that these cells are not dependent on CSF1 for their survival (222).

In the mouse brain, skin, liver, kidney, lung, and heart macrophages derive from embryonic precursors from the yolk sac and fetal liver and maintenance through local proliferation and self-renewal in adulthood in the absence of inflammation is independent of circulating monocytes (213, 217, 226-228).

However, intra embryonic transplantation of bone marrow progenitors into chicken embryos prior to hematopoiesis resulted in the production of long-lived tissue resident macrophages. In contrast, macrophages derived from yolk sac were not retained in the adult bird (229).

Nevertheless, in many adult murine tissues the resident macrophage population is a mix of cells derived from embryonic precursors and circulating monocytes (213). In the mammalian gut, dermis, heart and pancreas they seem to lack local proliferation and thus are replenished by monocytes from the blood circulation (218). However, intestinal macrophages in mammals

have been found to consist of several subsets originating from embryonic as well as hematopoietic cells (230, 231). Independent of their origin in the gut their maintenance seems to be dependent on live microbiota in mammals (230, 232). This seems to be dependent on the gut region since the replenishment of blood-derived macrophages in the small intestine is not altered in germ-free mice compared to specific pathogen-free mice. Thus in the small intestine, macrophage regulation seems to be more diet dependent (232).

In the chicken, Tomal et al. found almost no quantitative differences in the cecum of two week old germ-free and conventionally housed chickens (215). They are present in the gut and fully functional at hatch (55).

They are relatively long-lived cells. Intestinal macrophages have an estimated half-life of 4-6 weeks. Persistence for 8 month is described for mammalian intestinal macrophages (230). Cardiac macrophages derived from monocytes seem to have a longer half-life (8-12 weeks) as compared to dermis and intestinal macrophages (218).

2.3.2 Functions

More than 200 types of cells can be found in the (human) body and macrophages represent the most versatile cells in animals. These cells display also an extraordinary degree of autonomy (217). The evolution of multicellular organisms with increasing complexity of organs and tissues might have contributed to the repertoire of macrophage functions (217).

The importance and independence of the innate immune response is also highlighted by the fact that more than 95% of animals lack T-cells and yet survive in a sea of pathogens (233).

Quantitative immunological studies reveal that macrophages, which are ubiquitous cells make up 10% of immune cells in humans, but, due to their size, contribute almost 50% of the total cell mass. Lymphocytes make up 40% of the total number of immune cells and 15% of the mass and are located mainly in the lymph nodes and spleen. Neutrophils make up a similar proportion of the number and total mass of immune cells, with most neutrophils located in the bone marrow (234). In mammals, the largest proportion of macrophages can be found in the intestine (91). In chickens, not all body regions appear to be populated with macrophages. In this species, macrophages in the peritoneal cavity are largely absent (235).

Macrophages protect their vertebrates in several ways: regulating tissue homeostasis, wound healing and tissue remodeling as well as antigen presentation and protection against invading pathogens belongs to their protective repertoire (236). To fulfill all these functions, macrophages display a high plasticity. This diversity is imprinted by their ontogenetic origin, the organ context, status of activation or deactivation in a microbial context, tissue damage

and metabolic derangement and polarization of adaptive T-cell responses (213). Thus, important immunological and non-immunological functions can be attributed to those cells (217).

2.3.2.1 M1 / M2 macrophages

Macrophages are involved in all stages of inflammation and their extraordinary plasticity allows them to play key roles in the onset, maintenance and resolution of inflammatory processes (237, 238). Current therapeutic strategies aim to influence the polarization of macrophages (239). The most extreme phenotypes are M1 (pro-inflammatory) and M2 (anti-inflammatory) polarized.

In 1990 Abramson and his colleagues recognized that interleukin (IL)-4, which was mainly produced by Th2 cells, converted macrophages into a special activation state compared to IFN-y induced activation. With the discovery of up-regulation of macrophage mannose receptor as a specific marker of IL-4/IL-13-activated macrophages in 1992, the concept of alternatively activated macrophages (M2) was first proposed (233, 240).

M1 macrophages are recruited to the site of infection to eliminate the pathogen and participate in the Th1 response (87, 236). M2 macrophages contribute to Th2 mediated responses are more heavily involved in promoting tissue repair and limiting inflammation in chronic infections (87, 236). Anti-inflammatory molecules such as transforming growth factor beta (TGF-β), epidermal growth factor, and vascular endothelial growth factor are involved in this wound healing process (236) besides other molecules such as immunoresolvents (241, 242). Moreover, macrophage polarization shifts towards the M2 phenotype at the end of an infection (243). In addition, the formation of giant cells due to infections with for example foreign bodies often results in granulomas (244, 245). M1 macrophages can be found in early appearing granulomas. The M2-phenotype dominates in late granulomas (244).

It turned out that the killing and repair paradox was inter alia due to the ability of macrophages to convert arginine to either nitric oxide (NO) or ornithine, respectively (233). Wculek et al., Liu et al. and Thibaut et al. reviewed the metabolic pathways including glycolysis and oxidative phosphorylation involved in macrophage polarization (87, 246, 247). The fate of macrophages depends inter alia on different environmental conditions. Transcriptional, epigenetic and post-transcriptional pathways are involved in this process that drives polarization to one of the classically triggered pro-inflammatory M1 responses or M2 immune responses (211). Chen et al. summarized key factors regulating the polarization and reprogramming of M1 and M2 macrophages in mammals (211). Wang et al. reviewed molecular key-mechanisms influencing macrophage polarization and phenotype switch (242).

In addition to IL-4/IL-13, a variety of partially opposing stimuli, such as antibody immune complexes together with lipopolysaccharide (LPS) or IL-1, TGF-β, glucocorticoids and IL-10, were found to have the ability of alternative activation of macrophages (211). Mixed phenotypes can be found in several pathologic conditions such as tumors, neurodegenerative diseases and atherosclerotic plaques (242). Pro-inflammatory metabolites such as NO or lactate generated by macrophages themselves due to an external stimulus have also been shown to drive macrophage polarizations towards the anti-inflammatory phenotype in mammals (211). This seems to be consistent with Quiros et al. who suggested that the resolution of inflammation at mucosal sites is initiated by the pro-inflammatory environment (248).

Studies referring to M1 and M2 dichotomy in chickens are scarce. He et al. found that IL-4 reduced NO response and increased reactive oxygen species in macrophages challenged with LPS (249). Interestingly, in this study, IL-4 has also been demonstrated to induce NO production, when applied alone.

Chaudhari et al. showed that IL-4 induced expression of genes associated with an M2 phenotype. In addition, arginase activity was also enhanced. Furthermore, LPS treatment resulted in enhanced IL-4 production in chicken HD11 cells in this study (250). This is consistent with Sunday who used a similar approach (236). These findings suggest that IL-4 has potential anti-inflammatory properties depending on the experimental setting, although the exact mechanism remains unknown (249). Nevertheless, chicken monocyte derived cells primed with a combination of IL-4 and beta-glucan microparticulates (M-ßG) displayed higher NO levels than LPS, M-ßG or IL4 alone after repeated LPS stimulation (251).

Moreover, studies with Salmonella spp. infections in the chicken revealed severe metabolic changes resulting in a more M2-like polarized phenotype (252, 253).

Further studies are required to develop potential therapeutic strategies to regulate macrophage metabolism and to investigate the M1/M2 paradigm in birds.

2.3.2.2 Regulating homeostasis

The term homeostasis is composed of the ancient Greek terms *ὅμοιος* (Hómoios, “similar, equal, equal, equal”) +-stasis *στάσις* (stásis, “standing, state”) and comprises every self-regulating process through which biological systems tend to maintain their stability and at the same time adapt to conditions that are optimal for survival (254).

The stability achieved is actually a dynamic balance in which continuous changes take place and yet prevail relatively uniform conditions (255).

As Metchnikoff discovered from his work as a developmental embryologist, these cells display their phagocytic properties not only under inflammatory circumstances (210). Macrophages have long been recognized as important immune effector cells, but their primary role is to eliminate damaged cells and cell debris through their “janitorial” functions (256).

2.3.2.2.1 Tissue homeostasis

In the past, research focused on triggering inflammation with less attention and thus understanding how to resolve inflammation. Several biological aspects contribute to the resolution of inflammation including the elimination of heterophils through apoptosis induction and efferocytosis by macrophages or efflux from the tissue and the conversion of macrophages into alternatively activated promoting tissue healing (257).

Efferocytosis, a process by which macrophages eliminate dying cells, is an essential part of tissue homeostasis, control of inflammation, and repair of infected tissues (243). During the embryonic phase these cells can be found in areas of programmed cell death in both mammals and chickens (258).

In adults, millions of cells die every day in the human body and the constant replacement of cells and restoration of the macrophage-controlled extracellular matrix is fundamental to the health of every tissue in the body (217). For instance, without the recycling of iron and hemoglobin from approx. $2x10^{11}$ erythrocytes per day the host would be unable to survive (256). Scavenger receptors, phosphatidyl serine receptors, the thrombospondin receptor, integrins and complement receptors are involved in this process, which recognizes damage associated molecular patterns (DAMPs) (256, 259). Without harming the host, these damaged cells are recognized by macrophages and subsequently removed (236, 256).

In contrast, cellular debris caused by necrosis is often laden with endogenous danger signals such as heat shock proteins and nuclear proteins, histones, DNA, extracellular matrix components and phagocytosis causes alterations in macrophage physiology. Pro-inflammatory cytokines are produced and surface expression markers are altered (256). For example. Toll like receptors, intracellular pattern recognition receptors (PRRs) and the IL-1 receptor are involved in this process. Downstream signaling pathways include myeloid differentiation primary response protein 88 (MyD88). Detecting necrotic components makes macrophages one of the most important danger sensors in the body. Sensing and eliminating necrotic components can occur without involving lymphocytes (256).

2.3.2.2.2 Gut homeostasis

Especially at barrier tissues such as the gut, maintaining homeostasis is challenging. Here, cells of the innate and adaptive immune system constantly encounter a plethora of foreign antigens such as food, microbes and microbial derived metabolites (17). The intestine harbours the greatest bacterial load (232) and bacteria, and bacterial derived products translocate in the mammalian (125) as well as in the chicken gut (127, 128). On the other hand, in mammals, the largest proportion of macrophages can be found in the gut (91) which highlights their importance in this region.

The intestinal immune system must reach a delicate balance between destroying dangerous bacterial pathogens through resistance mechanisms while preserving the beneficial gut microbiota and tolerating food antigens.

Blaser et al., Pamer and Zheng et al. reviewed the fundamental role of the mucosal immune system in finding the balance between intestinal inflammations and gut homeostasis in mammals (104, 260, 261). Foster et al. summarized mutual control mechanisms (host to microbe, microbe to host and microbe to microbe) in mammals that are responsible to keep the stable association between host and microbiota (171). According to Miller et al., two components are involved in keeping the homeostasis of the gut microbiota-immune interactome. Besides intestinal defense mechanisms, the so-called microbiota nourishing immunity resembles another type of immune response. To keep a stable, diverse microbiota, which is important for the control of pathogenic bacteria, direct and indirect colonization resistance mechanisms together with a controlled lack of inflammation are required. In this concept specific interactions are involved (262).

In addition, according to Arias-Rojas et al. commensal encoded resilience mechanisms seem to be necessary during inflammatory processes in mammals (263).

The great plasticity of macrophages to respond to external stimuli includes tolerance mechanisms alongside defense strategies including innate immune memory (251, 264). Keeping homeostasis in the gut seems to require the full spectrum of macrophage functions alongside a finely balanced interkingdom signaling.

Jha et al. suggested that gut health is the interactome of mainly four components, including diet (i.e., nutrition), mucosa, microbiome, and the immune system working integrally together, to keep the intestinal homeostasis (265).

A comprehensive review on gut health from the perspective of nutritionists, physiologists, immunologists, and veterinarians is not yet available (130).

Medzhitov et al. illustrated the evolutionary values of tolerance as a defense mechanism to limit tissue damage caused by inflammation and therefore contribute to tissue homeostasis (266). In contrast to central (thymic) tolerance, which aims to avoid inadequate immunological reactions to the body's own structures, peripheral tolerance mechanisms, such as oral tolerance in the intestine and other mucosal surfaces play an important role against foreign antigens.

The mucosal immune system in mammals is inherently tolerogenic and stimulation with antigens has substantial regulatory systemic effects (267, 268). The discovery of oral tolerance is almost as old as the discovery of phagocytosis. In 1909 Alexander Besredka, an assistant of Metchnikoff and father of the “desensibilization- and oral immunization-methods” showed that the ingestion of milk prior to intracerebral injection rendered guinea pigs refractory to anaphylaxis. In the 1980s several studies proved that the oral route was capable to inhibit inflammatory diseases in animal models and currently oral tolerance has been successfully demonstrated in different autoimmunity and inflammatory diseases in mammals (269, 270). Other forms of immunotherapy have been reported using the nasal, sublingual and epidermic routes with variable results (269). Feeding an animal with proteins often results in immune non-responsiveness characterized through terms of dosage, mechanism, cell types involved and cytokine expression (271). Friedman summarized main observations on mechanisms of oral tolerance in birds. In the chicken as well as in mammals, tolerance can also be induced. Interestingly, administration of antigens to chickens less than 3 days old can induce systemic tolerance. On the other hand, maternal antibodies might help to prevent tolerance towards pathogens by preventing their interaction with induction mechanisms (271).

Immune cells of the myeloid and lymphoid cell line present in the intestine ensure a tolerogenic environment through various mechanisms. At the cellular level, intestinal immune homeostasis in mammals is controlled by a specific type of Treg cells, dendritic cells and macrophages (272, 273). Circulating Treg cells that have been primed in the intestine and subsequently encounter their antigen in the bloodstream may be responsible for the phenomenon of oral systemic tolerance (274, 275). Several anti-inflammatory molecules such as TGF-ß (276), IL-10 (277-279), prostaglandine E2 (280, 281), retinoic acid (282-284), vasoactive intestinal peptide (285, 286), thymic stromal lymphopoietin (287, 288), zymosan (289), niacin (290) and SCFAs (282) are involved in this process. Similar mechanisms are suspected in the chicken. TGF-ß4 is expressed in the chicken and shares similar anti-inflammatory functions (291). Following infections with coccidia TGF-ß in the chicken particular increases in the intestine and spleen (292). IL-10 is also expressed by a number of cells in this species (291, 293).

Macrophages represent key elements of the intestinal immune system and play an important role to maintain gut intestinal homeostasis. Through a combination of immune regulatory, anti-

bacterial and phagocytic functions they facilitate the homeostasis in the gut (294). In the chicken macrophages are present and functional at hatch (55).

In the mammalian intestine, they represent a unique phenotype, suppressing responses to bacterial infections for protection of the intestinal tissue from excessive inflammation (295). Through their interaction with the enteric nervous system, they also regulate gut secretion and motility (295).

Forming transepithelial dendrites, intestinal macrophages are capable to sample the lumen via opening the epithelial tight junctions (232).

In contrast to other tissue resident macrophages, they contribute to the production of Tregs and Th17 cells through their constitutive expression of IL10 (91). Although, the high expression levels of IL-10 in the intestine mainly depend on the production by T-cells (215). IL-10 is also produced by intestinal dendritic cells and deletion of the IL-10 receptor on intestinal macrophages leads to spontaneous severe colitis in mice and promotes IBD in pediatric patients (294). The hyporesponsiveness of TLR-stimulation observed in intestinal macrophages might also be due to the high IL-10 concentrations in the gut (232). Interestingly, in mammalian species, the production of IL-10 in response to Salmonella infection has been observed in macrophages and certain B-cell subtypes via TLR4 recognition of LPS (296, 297).

Moreover, intestinal macrophages are highly phagocytic but seem to omit upregulation of pro-inflammatory cytokines after uptake of pathogens (91). The expression level of pro-inflammatory cytokines such as iNOS, IL-6, TNF-α seems to be low in mammalian intestinal macrophages (232). Moreover, human intestinal macrophages don´t produce nitric oxide upon ingestion of bacteria (298). Bain et al., Ruder et al. and Hegarty et al. reviewed the functional properties of intestinal macrophages in mammals (232, 299, 300).

In addition, in mammals bacterial translocation seems to be due to a lack of these lamina propria macrophages rather than to a defective epithelial barrier (232). It remains unclear whether lamina propria macrophages transport bacterial antigens to mesenteric lymph nodes. Even under inflammatory conditions these cells don´t seem to migrate to lymph nodes (232). Interestingly, under experimentally evoked dysbiotic conditions or chronic colitis they change their habits and invade lymph nodes (232). Food antigens were also shown to be taken up by CX3CR1+ macrophages, followed by presentation to dendritic cells (232).

Studies with germ-free mammals demonstrate the regulation of the intestinal macrophage pool in the large intestine by microbiota. Thus, germ-free animals display reduced numbers of intestinal macrophages (232). This is in contrast to Tomal et al., who found almost no quantitative differences in the cecum of two week old germ-free and conventionally housed chickens (215).

The great plasticity of macrophages to respond to external stimuli includes tolerance mechanisms alongside defense strategies including innate immune memory (264, 301). Metabolic switches from glycolysis to oxidative phosphorylation seem to be involved in this process (233, 302). Thus, a disequilibrium of M1/M2 polarization is referred to several immunological diseases in the gut (303-305).

In the chicken, Tomal et al. found that the expression of several genes including TLR4 was upregulated in macrophages of conventionally housed chickens compared to germ-free animals at homeostasis. Unexpectedly, additional infection with Eimeria tenella displayed a downregulation of several inflammatory mediators including TNF-α in GF chickens compared to conventionally housed animals. The addition of microbiota several days after infection partially restored the macrophage response and recruitment. Moreover, the expression levels of IL-10 in macrophages of infected animals were increased (215). This is in line with findings in the mammalian literature on Salmonella infections (306). This pro- and anti-inflammatory response might be due to different macrophage subpopulations in the ceca, similar to mammals (232) and could be a part of the immune surveillance (306). In 2022, Boodhoo et al. also recognized differences in NO production in macrophages derived from different sections of the small intestine. Compared to jejunal, ileal and cecal macrophages, duodenal macrophages did not significantly respond to Clostridium perfringens treatment. The addition of Lactobacillus lactis lead to enhanced NO levels in this gut section (307). The chicken HD11 cell line has been found to express IL-10 upon stimulation with LPS alongside pro-inflammatory molecules (293).

Potentiated or suppressed immunological reactions can be observed in macrophages after re-exposure to a stimulus in mammals (302). In addition, immunological training has been demonstrated to exert enhanced reactions in mammals (264).

In the chicken, Verwoolde et al. demonstrated that monocytes responded to repeated stimuli with enhanced NO production. Additionally, differences could be observed in broilers and layers concerning expression of surface markers and NO production in monocyte derived cells following repeated stimulation, suggesting genetic differences within these species (251). Interestingly, in bone marrow derived cells the NO enhancing effect was absent in layers (251) and IL-10 production was not induced in broilers (301). Repeated LPS stimuli failed to induce tolerance in chicken monocytes in this study. However, repeated stimuli have been found to evoke ameliorated immunological responses in both chicken (308, 309) and mammalian (310, 311) macrophages in *vivo* and in *vitro*. This process is also called endotoxin tolerance (312). Hypo- or hyperresponsiveness could also be due to a different genetic background, since NO levels in LPS treated chickens of different breeds have been demonstrated to differ significantly (313, 314). Differences in TLR4 expression levels could be causative to this phenomenon

(313). Age-related immunological differences could also play a role in mammals (315). Moreover, tolerogenic and enhanced immunity depends on the magnitude and duration of stimulation in mammals (316). Current knowledge of trained vs tolerant immune mechanisms is reviewed by Lajqi et al. (317).

Further research is needed in this area to decipher the complex interactions between microbiota and the innate immune system.

2.3.2.3 Antimicrobial properties

Located in a strategically optimal position under epithelial barriers, macrophages often represent the first line of defense. Macrophages act as sensor cells of the innate immune system and their eponymous, probably evolutionary most conserved function is phagocytosis (318). Moreover, in both mammals (319) and chickens (320) these cells are highly motile and crawl through the tissue following chemotactic stimuli, thus they are rapidly recruited upon infection (321, 322). In mammals, three- dimensional amoeboid and mesenchymal locomotion have been described, seemingly dependent on tissue structure (323).

2.3.2.3.1 Pattern recognition receptors

To recognize pathogenic invaders prior to phagocytosis, these cells are equipped with a plethora of intra and extracellular pattern recognition receptors. These receptors enable macrophages, to recognize for instance opsonized and non- opsonized microbes or microbiota derived components also called pathogen-associated molecular patterns (PAMPS) such as LPS derived from Gram neg. bacteria. These PAMPs are phylogenetically conserved and provide an initial discrimination between self and non-self (324). For example, Toll-like receptors, nucleotide binding receptors, scavenger receptors, complement receptors, C-type lectin and mannose receptors are involved in this process. In addition, G-protein coupled receptors such as the folate receptor, adenosine receptor or purinergic receptor recognize inter alia bacterial polypeptides in mammals. Activation of these receptor systems results in phagocytosis and destruction in most cases (217).

The PRR system is also present in chickens, although not completely identical to the human system. For example, chicken TLRs share different amino acid sequences with their human counterpart. Not all TLRs found in humans were also detected in the chicken up to now (325).

Ongoing research reveals further receptors in this species (325). In the chicken functional studies indicate the presence of some of these molecules on macrophage cell lines as well as on primary macrophages (326).

MRC1L-B, a mannose binding receptor recognized by the KUL01 monoclonal antibody is used to identify monocytes and macrophages in the chicken. KUL01mAb was developed by Mast in 1998 and also recognizes Langerhans cells (16).

Chicken Toll-like receptors were reviewed by Nawab and Neerukonda (327, 328). In the present study the chicken TLR4 system is used to investigate the effects of SCFAs on chicken macrophages. The mammalian and chicken lineages diverged 300 million years ago and the avian TLR repertoire shares orthologues and distinct new genes (329). TLR4 is highly expressed on chicken macrophages and shares approx. 44% amino acid identity with the human counterpart (330). In the chicken as well as in mammals LPS represents the ligand for this receptor (327). Several downstream signal transduction pathways are involved in the TLR4 system including myeloid differentiation primary response protein 88, TIR domain containing adaptor protein, TIR domain-containing adaptor protein inducing interferon beta, translocating chain-associated membrane protein (TRAM) and selective androgen receptor modulator in mammals. In the chicken TRAM seems to be absent (16, 331). Downstream pathways in mammals include IL-1 receptor-associated kinase, Transforming growth factor beta-activated kinase, Tumor necrosis factor receptor associated factor 6 (332) mitogen-activated protein (333) Bruton's tyrosine kinase (334) IkappaB kinases, Interferon regulatory factors family members (335) as well as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) (336-338) signaling.

Binding to this receptor subsequently results in upregulation of numerous pro-inflammatory molecules or microbicidal metabolites such as TNF-α, IL1-ß and IL-6; IL-8 (338) and nitric oxide, iNOS amongst many others including antimicrobial peptides (339). RNA-Seq studies are further required to enable a more thorough insight into chicken macrophage antimicrobial responses.

2.3.2.3.2 Phagolysosomes and respiratory burst

After recognition, downstream effector mechanisms further include the formation of phagosomes with subsequent fusion with lysosomes in mammals as well as in chickens (340) Lysosomes contain several antimicrobial proteins and enzymes (341). The acidification in matured phagolysosomes (pH 5) serves several bactericidal mechanisms including enzyme

activation and enhancement of the production of reactive oxygen species, a process also called respiratory burst (342).

During this process oxygen is reduced to superoxide (O_2-) with the help of the Nicotinamid adenine dinukleotid phosphat (NADPH) oxidase. Superoxiddismutase further catalyzes the reaction of O_2- with itself to form hydrogen peroxide (H_2O_2). The catalytic decomposition of hydrogen peroxide produces the highly reactive hydroxyl radical (·OH), which can attack a large number of molecules. H_2O_2 can easily penetrate membranes and is further transformed to Hypochlorous acid (HOCl) a highly reactive oxidant, displaying bactericidal and virucidal effects (343, 344).

For example, different Salmonella serotypes in the chicken induce respiratory burst activity (345-347).

2.3.2.3.3 Nitric oxide production

Another mechanism, which directly attacks pathogens is mediated by nitric oxide (NO) production in both mammals and chickens (348, 349).

In 1991, Sung et al. were the first to demonstrate L-arginine dependent NO production by chicken macrophages (350).

The hallmark of classically activated macrophages (M1) is the production of NO. The microbicidal effects of nitric oxide (NO), an endogenously produced free radical secreted by macrophages were identified in 1987. Its bactericidal effects include both oxidative and nitrosative stressors such as lipid peroxidation, nitrosation of membrane proteins as well as DNA damage (351). Thus, it interferes with bacterial proliferation. Nevertheless, its functions are not limited to antibacterial properties and many other cells such as neurons and endothelial cells are capable of NO production in mammals as well as in chickens (349).

The nitric oxide synthase, which is the enzyme responsible for NO production appears in three isoforms. The neuronal isoform (nNOS) is expressed in neurons and NO functions as a signaling molecule for example in neurotransmission. The endothelial form (eNOS) is expressed by vascular endothelial cells and regulates the vascular tone. eNOS and nNOS are constitutively expressed at low levels in the chicken (349). In contrast, the expression of iNOS is inducible and the production of NO by iNOS is classified as “high-output”. iNOS is expressed in macrophages in response to inflammatory stimuli following for example TLR4 signaling (349). iNOS expression as well as NO production is examined in this study to investigate the effects of SCFAs, mainly butyrate on chicken macrophages.

Nitric oxide synthase catalyzes the reaction of L-arginine to L-citrulline and NO. NO is quickly converted into NO_2- and NO_3- at a ratio 3:2. Thus, direct measurement of NO is quite challenging. In contrast, NO_2- can be detected and quantified by the Griess reaction, a method frequently used in avian and mammalian macrophage research (339, 348, 352).

In contrast to mammals, the chicken lacks carbamoyl phosphatase synthase I (349, 353). In addition, ornithine transcarbamylase I is expressed at lower levels in this species (349). Therefore, nutritional supply of arginine is necessary for this metabolic pathway (349). Another amino acid, tryptophan is involved in the synthesis of nicotinamide and therefore also interferes with NO production in the chicken (354). Tetrahydrobioperin, synthesized from guanosine triphosphate is also required in this process (355). Moreover, use of NFk-B inhibitors demonstrated the involvement of NF-kB in the induction pathway for NO synthase in the chicken (337, 356).

Nevertheless, in the chicken as well as in mammals, NO production is not limited to stimulation of the TLR4 system. For example, lipoteichoic acid, a potent TLR2 agonist induced NO in neonatal chicken blood monocytes. In contrast, Pam2CSK, a synthetic lipoprotein and potent TLR2 agonist displayed almost no NO production (357).

In addition, CD40 ligand has also been demonstrated to induce NO production (358). Moreover, IL-4 a M2 macrophage inducer in mammals, induced NO production in chicken macrophages. This effect was absent, when combined with different TLR agonists (236, 249, 250). Further research is needed in this area to investigate the interfering molecular mechanisms in chicken macrophage NO release on a molecular level.

2.3.2.3.4 Cytokine production

Binding of microbial components to PRRs triggers fast direct and relatively unspecific effector mechanisms such as phagocytosis, degradation and killing (213, 217).

In both mammals and chickens, as a prerequisite to a successful immune response macrophages recruit immune cells of the adaptive branch and present antigens via MHC class I molecules and MHC class II molecules to enhance a more specific pathogen regulation (359).

On the other hand, these pro-inflammatory processes need to be regulated and coordinated bidirectionally to prevent excessive and destructive inflammatory processes (360).

The biological effect depends on the mediator molecule itself and the target cell. Cell activation, proliferation, polarization, apoptosis and migration can be influenced (361).

Besides the expression of iNOs, in this study IL1ß, IL-6, IL-8, TNF-α are investigated in chicken splenic macrophages. Lacking lymph nodes, the spleen is considered to be the most important secondary lymphoid organ in this species and is often involved in humoral and cellular immune responses (340, 362). Most of these cytokines are rapidly produced as a uniform response to microbial stimuli often as part of an acute phase reaction (363).

IL-1ß has been shown to exert pro-and anti-inflammatory properties in mammals. For example, it is involved in induction of fever, induction of B-cell maturation and function, elevation of corticosterone levels through neuronal stimulation of adrenocorticotrope hormone, prostaglandin E2 production and promotion of IL-6 expression. On the other hand, anti-inflammatory reactions upon IL-1ß stimulation have been observed in humans (364).

Chicken IL-1ß shares 25% sequence homology with the human orthologue. A variety of different cell types has been shown to express IL-1ß in this species in response to bacterial, viral and parasitic infections including the macrophage cell line HD11 (365). IL1-ß has been shown to exert antibody inducing effects as a vaccine adjuvant in this species (366).

A plethora of functions can be attributed to IL-6. This cytokine is produced by many cell types and is known to have important roles in the immune response, inflammation and hematopoiesis in mammals. It stimulates B-cells to differentiate into antibody producing plasma cells, induces T-cell growth and differentiation as well as differentiation of macrophages (367, 368). In the chicken IL-6 levels rapidly increase due to H5N1 infection in different organs including the spleen (369, 370).

TNF-α, a member of the tumor necrosis factor superfamily, shares many of its functions with IL-1ß and IL-6. It plays important roles in immune defense reactions, lymphocyte homeostasis, tissue development and inflammation (371). Upon LPS stimulation it is rapidly induced in chicken primary macrophages derived from blood monocytes or spleen (372).

In contrast to humans, the chicken appears to have evolved two CXCL8-like chemokines (IL-8L1, IL8-L2) (373). This protein functions as a chemoattractant and recruits other immune cells, which subsequently participate in the response. The chicken IL-8 has been demonstrated to play an important role in recruitment of different immune cells in an infection with Salmonella enteritidis (374) and Eimeria species (375).

3 AIM

The functional repertoire of macrophages comprises pro-and anti-inflammatory features and is regulated by environmental factors.

In addition, macrophages are closely linked to the adaptive immune response through the production of signaling molecules. They play an essential part in the onset, maintenance and resolution of inflammation.

Therefore, regulation of macrophage function is described as a key factor in regulating immune answers.

In the chicken, the effects of SCFAs, especially butyrate were tested on different macrophage cell lines, monocyte and bone marrow derived cells. As far as we know, there is no scientific work examining the effects of SCFAs, particularly butyrate, on splenic and intestinal macrophages. Our aim was to add to the chicken literature considering the effects of SCFAs on avian macrophages derived from different tissue sources including the gut.

4 MATERIAL AND METHODS

4.1 NO-Assay

Material:

Griess solution A (storage 4°C)	
Sulfanilamide (storage RT)	**(Sigma Aldrich GmbH)**
Phosphoric acid 2,5% (storage RT)	**(Applichem GmbH)**
Griess solution B (stored at 4°C)	
Naphtyethylendiamine (storage RT)	**(Sigma Aldrich GmbH)**
Phosphoric acid 2,5% (storage RT)	**(Applichem GmbH)**
Sodium nitrite 1mM in aqua dest. (storage 4°C)	**(Applichem GmbH)**
96-well flat bottom plate	**(Thermo Fisher Scientific, Inc.)**
Sunrise Microplate Reader	**(Tecan Trading AG)**
Cell culture medium	**(described under 4.3)**
Analytical scale	**(Mettler AE 100, Mettler Toledo GmbH)**

The Griess test is a widely used method to detect and quantify nitrite anions in solutions. Two consecutive reactions are involved in this test. First, the nitrite anion reacts with the sulfanilamide to form a diazonium salt. Subsequently, a pink-red azo dye is formed with N-(1-naphthylethylendiamine) in an azo-coupling reaction. Spectrophotometric quantification is possible based on color intensity.

All reagents were used at room temperature. Griess solution A was prepared by adding 1g of sulfanilamide to 100ml of 2.5% phosphoric acid. The Griess solution B was prepared by adding 0.3g of naphtyethylenediamine to 100ml of 2.5% phosphoric acid. Both solutions were stored at 4°C. To measure the nitrite content in the cell culture supernatant, both solutions were first mixed in equal parts and used at RT. 50µL of cell culture supernatant was removed and transferred to a nonsterile 96-well plate. The mixture of Griess solution A and B was then added in equal parts to the cell culture supernatant. Sodium nitrite was dissolved in aqua dest. to a final concentration of 1mM. A standard curve was created for each measurement by titrating the 1mM sodium nitrite solution in a log2 scale in the respective cell culture medium. The plate was shaken for 5 seconds in the plate reader and subsequently measured at 540nm measurement wavelength/620nm reference wavelength. The nitrite value of the samples was calculated from a calibrated standard curve (linear regression) using sodium nitrite ranging from 0 to 500µM. In order to be able to calculate statistics (SPSS 29, IBM), zero values were set to 0.1. Graphical representations were created using GraphPad Prism 5.

4.2 SCFA- solutions

Material

Sodium butyrate (storage RT)	**(Sigma Aldrich GmbH)**
Sodium propionate (storage RT)	**(Sigma Aldrich GmbH)**
Sodium acetate (storage RT)	**(Sigma Aldrich GmbH)**
Sterile syringe filter 0.22µm	**(Sarstedt AG &Co. KG)**
10ml syringe	**(B Braun AG)**
Analytic scale	**(Mettler AE, Toledo GmbH)**

A 200mM standard solution of each SCFA was prepared in the corresponding cell culture medium. For this purpose, SCFAs were dissolved in the appropriate cell culture medium. Sterile filtration was carried out under aseptic conditions and the solutions were stored at 4°C.

4.3 Cell culture media

Unless otherwise stated, cell culture media listed below were stored at 4°C.

HD11 cells- RPMI 8/2

445ml RPMI 1640 with Glutamax	**(Thermo Fisher Scientific, Inc.)**
40ml fetal bovine serum (FBS)	**(Thermo Fisher Scientific, Inc.)**
10ml chicken serum (CHS)	**(Thermo Fisher Scientific, Inc.)**

RAW 264.7 cells- RPMI 10

445ml RPMI 1640 with Glutamax	**(Thermo Fisher Scientific, Inc.)**
40ml FBS	**(Thermo Fisher Scientific, Inc.)**

Primary cells- RPMI 8/2/1

445ml RPMI 1640 with Glutamax	**(Thermo Fisher Scientific, Inc.)**
40ml FBS	**(Thermo Fisher Scientific, Inc.)**
10ml CHS	**(Thermo Fisher Scientific, Inc.)**
5ml Penicillin (10000 I.U.)/Streptomycin (10mg/ml)	**(Sigma Aldrich GmbH)**

Organ collection medium- RPMI 1640 1%

RPMI 1640 with Glutamax	**(Thermo Fisher Scientific, Inc.)**
5ml Penicillin (10000 I.U.)/Streptomycin (10mg/ml)	**(Sigma Aldrich GmbH)**

4.4 Cell lines

4.4.1 Thawing

Material:

50ml tubes	**(Sarstedt AG &Co. KG)**
RPMI 1640 (storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
Cell culture medium	**(described under 4.3)**
10ml cell culture flask (25cm^2)	**(Sarstedt AG &Co. KG)**
DPBS (phosphate buffered saline, pH 7,2) (37°C)	**(Thermo Fisher Scientific, Inc.)**

The frozen cryovials were thawed by swirling in a water bath at 37°C and transferred to a 50ml sample tube as quickly as possible. 10ml RPMI 1640 medium (°4C) were added drop by drop whilst gently swirling to eliminate the cytotoxic dimethyl sulfoxide (DMSO). Subsequently, the tube was quickly filled with RPMI 1640 (4°C) and centrifuged for 10 minutes at 225×g at 4°C. The cells were then washed twice in PBS (37°C), resuspended in the respective cell culture medium, and seeded in 10ml flasks. Dependent on the cell type, the cells were cultured at 40°C or 37°C as described in 4.4.2.1 and 4.4.3.1. The cell culture medium was replaced by fresh medium the following day.

4.4.2 HD11 cell line

4.4.2.1 Culturing

Material:

DPBS (pH 7,2, storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
DPBS-EDTA (storage 4°C)	
50µl EDTA (0.5M) (storage RT)	**(Thermo Fisher Scientific, Inc.)**
50ml PBS (pH 7,2, storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
RPMI 8/2	**(described under 4.3)**
Eppendorf Centrifuge 5810R	**(Eppendorf AG)**
50ml tubes	**(Sarstedt AG & Co. KG)**
Cell culture flask (75cm^2)	**(Sarstedt AG &Co. KG)**

3 days after thawing, cells were transferred from the 10ml flask to a 75cm^2 cell culture flask and incubated at 40°C and 5% CO_2. Every 2 to 3 days cells were split at a ratio of 1:3 using DPBS-EDTA (5ml) for detachment after washing with PBS (10ml; 37°C). Since the cells adhere

very firmly, the bottom of the cell culture flask was additionally tapped. Incubation with EDTA lasted no longer than 5 minutes. The EDTA reaction was stopped using RPMI 8/2 (RPMI 1640 supplemented with 8% FBS and 2% CHS; 37°C) at a ratio 2:1. Subsequently cells were washed (20°C, 10min, 222xg) and resuspended in cell culture medium.

4.4.2.2 Stimulation

Material:

LPS (Salmonella Typhimurium LPS) (storage 4°C)	**(Sigma Aldrich GmbH)**
Sterile 96 well flat bottom cell culture plate	**(Thermo Fisher Scientific, Inc.)**
RPMI 8/2	**(described under 4.3)**
Sodium butyrate 200mM (storage 4°C)	**(described under 4.2)**
Sodium propionate 200mM (storage 4°C)	**(described under 4.2)**
Sodium acetate 200mM (storage 4°C)	**(described under 4.2)**
Neubauer counting chamber	**(Brand GmbH & Co. KG)**
Trypan blue (storage RT)	**(Biochrom GmbH)**

After detachment as described under 4.4.2.1 the cell count was set to $1x10^6$/ml using a Neubauer counting chamber following live-death staining with trypan blue. Cells were seeded into sterile 96-well plates (100µl/well) and incubated for 24h at 40°C and 5% CO_2. In a first approach LPS (10ng/ml) was titrated separately in a log2 scale using a sterile 96 well plate. The cell culture supernatant of the HD11 cells was then removed and 100µl of the titration series was added to the cells in triplicate. Cells were incubated at 40 °C and 5% CO_2. The NO level was measured 24h later as described under 4.1.

This basic experimental setting was subsequently modified to examine the effects of SCFAs.

SCFAs (Na-butyrate,-propionate,-acetate) were adjusted to a final concentration of 4mM in the respective cell culture medium and subsequently applied to 96 well plates in triplicates (50µl/well). HD11 cells ($1x10^5$/well) were added at the same volume to the SCFAs, achieving a final SCFA concentration of 2mM and incubated for 24h at 40°C and 5% CO_2, followed by LPS (5ng/ml/0.31ng/ml/0.08ng/ml) stimulation for additional 24h in the presence of 2mM SCFAs in fresh cell culture medium. Nitric oxide production was assessed using Griess assay as described under 4.1 at different time points.

4.4.3 RAW 264.7 cell line

4.4.3.1 Culturing

Material:

DPBS (pH 7,2, storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
RPMI 10	**(described under 4.3)**
Eppendorf Centrifuge 5810R	**(Eppendorf AG)**
50ml tube	**(Sarstedt AG & Co. KG)**
Neubauer counting chamber	**(Brand GmbH & Co. KG)**
Trypan blue (storage RT)	**(Biochrom GmbH)**
Cell scraper 36cm	**(Sarstedt AG & Co. KG)**
Cell culture flask (75cm^2)	**(Sarstedt AG &Co. KG)**

Similar to HD11 cells, 3 days after thawing, cells were transferred to a 75cm^2 cell culture flask and incubated at 37°C and 5% CO_2. Every 2 to 3 days cells were split at a ratio 1:3 using a cell scraper for detachment. Subsequently cells were washed with DPBS (20°C, 10min, 222xg) and resuspended in cell culture medium.

4.4.3.2 Stimulation

Material:

LPS (Salmonella Typhimurium LPS) (storage 4°C)	**(Sigma Aldrich GmbH)**
Sterile 96 well flat bottom cell culture plate	**(Thermo Fisher Scientific, Inc.)**
RPMI 10	**(described under 4.3)**
Sodium butyrate 200mM	**(described under 4.2)**
Sodium propionate 200mM	**(described under 4.2)**
Sodium acetate 200mM	**(described under 4.2)**
Neubauer counting chamber	**(Brand GmbH & Co. KG)**
Trypan blue (stored at RT)	**(Biochrom GmbH)**

Based on the experiments with the HD11 cells, the cell count was set to 1x10^6/ml using Neubauer counting chamber following live-death staining with trypan blue.

Butyrate was adjusted to a final concentration of 4mM in the respective cell culture medium and subsequently applied to 96 well plates in triplicates (50µl/well). RAW 264.7 cells

($1x10^5$/well) were added 1:1, achieving a final concentration of 2mM. LPS (10ng/ml) was separately titrated in the absence or presence of butyrate 2mM in a log2 scale using a sterile 96 well plate. The cell culture supernatant of the RAW 264.7 cells was then removed and 100µl of the titration series was added to the cells in triplicate. Cells were incubated at 40 °C and 5% CO_2. The NO level was measured 24h later as described under 4.1.

4.5 Isolation / culturing / stimulation of primary cells

4.5.1 Animals

White Leghorn line M11 chickens (Federal Research Institute for Animal Health, Neustadt, Germany) were hatched and housed under conventional conditions. Water and a commercial diet were provided ad libitum. At the age of 8-9 weeks, the chickens were euthanized for tissue collection by neck-blow with subsequent exsanguination.

4.5.2 Isolation

Organs were removed following stunning and exsanguination of the animals. All cell preparation steps were performed under aseptic conditions using the Microflow Advanced Biosafety Cabinet (Thermo Fisher Scientific, Inc.).

4.5.2.1 Monocyte derived macrophages

Material:

10ml syringe	**(B Braun AG)**
Needle (size 0.7 x 40mm)	**(Becton Dickinson GmbH)**
Heparinsolution	
5ml Heparin-Natrium (25.000 I.U./5ml)	**(ratiopharm GmbH)**
45ml RPMI 1640 with Glutamax	**(Thermo Fisher Scientific, Inc.)**
The solution was aliquoted and stored at 4°C	
50ml tubes	**(Sarstedt AG & Co. KG)**
Eppendorf Centrifuge 5810R	**(Eppendorf AG)**
DPBS (pH 7,2)	**(Thermo Fisher Scientific, Inc.)**
Histopaque solution (storage RT)	**(Sigma Aldrich GmbH)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**

Blood lymphocytes were collected in 10ml syringes prepared with 100µl of sterile heparin (500 I.U./ml). After sterile collection from the jugular vein (Gz.:55.2-1-54-2532.0-60-2015), heparinized whole blood samples were immediately transported to the laboratory.

All steps were performed at RT. Blood samples were diluted with DPBS at a ratio 1:1 and subsequently density centrifuged over an equal volume (10ml) of 1.077g/ml Histopaque solution (20°C, 650xg for 12min, without brake). The serum- and interphase were collected and washed with DPBS (20°C, 222xg, 20min). Subsequently cells were resuspended in RPMI 8/2/1.

4.5.2.2 Splenic macrophages

Material:

10ml syringe	**(B Braun AG)**
50ml tubes	**(Sarstedt AG & Co. KG)**
Eppendorf Centrifuge 5810R	**(Eppendorf AG)**
DPBS (pH 7,2, storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
Histopaque solution (storage RT)	**(Sigma Aldrich GmbH)**
RPMI 1640 1% (4°C)	**(described under 4.3)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**
Cell dissociation sieve	**(Sigma Aldrich GmbH)**
Petri dish	**(Thermo Fisher Scientific, Inc.)**

Organs were removed following stunning and exsanguination of the animals.

The spleen was collected, transferred to a 50ml tube containing RPMI 1% (4°C) and immediately transported to the laboratory. To prepare a single cell suspension, the spleen was homogenized using a syringe plunger and a metal cell strainer placed in a petri dish filled with PBS. The organ capsule was removed upon homogenization. The cell suspension was transferred to a 50ml tube. The 50ml tube was then refilled with DPBS (4°C) and the tissue debris was subsequently sedimented on ice for 10 minutes. The supernatant was transferred to a new 50ml tube and refilled with PBS. The cells were washed (20°C, 222xg, 10min) and subsequently density centrifuged over 1.077g/ml Histopaque separating solution (20°C, 650 xg for 12min, without brake). The upper layer and interphase were collected and washed with DPBS (20°C, 222xg, 20min).

4.5.2.3 Bone marrow derived macrophages

Material (sterile):

Bone saw	
10ml syringe	**(B Braun AG)**
Needle (0.7x40mm)	**(Becton Dickinson)**
50ml tubes	**(Sarstedt AG & Co. KG)**
Eppendorf Centrifuge 5810R	**(Eppendorf AG)**
DPBS (pH 7,2, storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
Histopaque solution (storage RT)	**(Sigma Aldrich GmbH)**
RPMI 1640 1% (4°C)	**(described under 4.3)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**
Cell dissociation sieve	**(Sigma Aldrich GmbH)**
Petri dish	**(Thermo Fisher Scientific, Inc.)**

The humerus and femur were collected, transferred to a 50ml tube containing RPMI 1% (4°C) and immediately transported to the laboratory. The bone was cut with a bone saw. Using a syringe-cannula combination and PBS, the bone marrow cavity was flushed onto a cell strainer placed in a petri dish.

To prepare a single cell suspension, the bone marrow was homogenized using a syringe plunger. After homogenization, the suspension was transferred to a 50ml tube and refilled with DPBS (4°C) and the tissue debris was subsequently sedimented on ice for 10 minutes. The supernatant was then transferred to a new 50 ml tube and refilled with DPBS. The cells were washed (20°C, 222xg, 10min) and subsequently density centrifuged over 1.077 g/ml Histopaque separating solution (20°C, 650xg for 12min, without brake). The serum- and interphase were collected and washed with PBS (20°C, 222xg, 20min). Cells were resuspended in RPMI 8/2/1.

4.5.2.4 Intestinal macrophages (ileum / cecum)

Material (sterile):

Button cannula	
20ml syringe	**(B Braun AG)**
50ml tubes	**(Sarstedt AG & Co. KG)**
Eppendorf Centrifuge 5810R	**(Eppendorf AG)**
DPBS (pH 7,2, 4°C)	**(Thermo Fisher Scientific, Inc.)**
Penicillin (10000 i.U.)/Streptomycin (10mg/ml) (stored at -20°C)	**(Sigma Aldrich GmbH)**
Histopaque solution (storage RT)	**(Sigma Aldrich GmbH)**
RPMI 1640 1% (4°C)	**(described under 4.3)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**
DTT (dithiotreitol)	**(Applichem GmbH)**
EDTA (Ethylenediaminetetraacetic acid)	**(Applichem GmbH)**
Collagenase D (storage 4°C)	**(Roche Deutschland Holding GmbH)**
HBSS (Hank´s balanced salt solution) (37°C)	**(Sigma Aldrich GmbH)**
Analytic scale	**(Mettler AE, Toledo GmbH)**
Scalpel	**(C. Bruno Bayha GmbH)**
Erlenmeyerflask	**(Sigma Aldrich GmbH)**
Cell dissociation sieve	**(Sigma Aldrich GmbH)**
Petri dish	**(Thermo Fisher Scientific, Inc.)**
100µm cell strainer	**(Thermo Fisher Scientific, Inc.)**
Bacteria shaker	**(Edmund Bühler GmbH)**

Both ceca and the intervening ileum were extracted from the body. Cecal tonsils were removed. The gut sections were immediately repeatedly filled and carefully but thoroughly cleaned with ice cold DPBS (approx. 250ml; 4°C) supplemented with 1% penicillin/streptomycin (DPBS 1%) using a syringe and a button needle until no more ingesta were seen through the translucent intestinal wall. The many washing steps were particularly necessary in the cecum to remove the past-like ingesta.

Intestinal sections were subsequently filled with ice cold sterile RPMI 1640 1% and transported to the lab in the same medium. Before removement of the epithelial layer both sections (ileum and cecum) were washed with sterile DPBS 1% (4°C; approx. 250ml used). To remove the

epithelial layer and the intra epithelial lymphocytes, gut sections were filled with prewarmed (37°C) sterile DPBS containing 1mM EDTA and 1mM DTT. The ends of the gut sections were tied up and the segments were placed in a sterile Erlenmeyer flask, covered with 37°C sterile DPBS 1% and incubated at 37°C for 30min, 120rpm in a prewarmed bacteria shaker. The cell suspension was discarded unless the IEL obtained in this way were used for other purposes. The intestinal sections were refilled with DPBS-EDTA-DTT and gently rubbed between the fingers to remove remaining epithelial cells. Subsequently, in order to prevent interactions of DTT and collagenase D, the gut sections were thoroughly washed with sterile DPBS 1% (approx. 200ml) as described above. Gut sections were filled with digestion solution containing 8mg collagenase D in 15ml prewarmed (37°C) HBSS, 5ml per gut section and incubated for 40min in an Erlenmeyer flask, covered with sterile prewarmed DPBS at 37°C in the bacteria shaker (120rpm). Stopping medium was prepared (4°C, RPMI 8/2/1, 2mM DTT) and section content was added in a ratio of 1:3. The intestinal sections were opened longitudinally and the lamina propria was scraped with a scalpel. The material thus obtained was placed on a 100μm cell strainer placed in a glass Petri dish filled with sterile DPBS (4°C) and gently rubbed through with a syringe plunger. The cells suspension was drawn up again with the syringe, spilled onto the cell strainer and rubbed through at total of three times. The cell suspension obtained in this way was then placed in the same tube with the stopping medium, distributed into two 50ml tubes, washed with sterile DPBS (20°C, 222xg, 20min), subsequently resuspended in RPMI 8/2/1 and finally density centrifuged over 1.077g/ml Histopaque solution (20°C, 650xg for 12min, without brake). After collecting the inter-and superior phase the cells were washed with DPBS 1% for 20min at 20°C and 222xg two times with a total of 200ml of sterile DPBS.

A total of $4.5x10^6$ cells were removed for Flow cytometric analysis. The respective tubes were refilled again and centrifuged at 222xg, 20°C for 20min. The many washing steps served to reduce the number of bacteria, which was necessary above all in the cecum.

The cells were then resuspended in RPMI 8/2/1 and counted using Neubauer counting chamber after staining with trypan blue.

4.5.3 FACS-staining of intestinal macrophages (ileum / cecum)

Material:

Single cell suspensions (storage 4°C)

Primary and secondary antibodies diluted in Fluo-buffer (storage 4°C)

Fluo-buffer (storage 4°C)

5g albumin, fraction V (storage 4°C)	**(Applichem GmbH)**
50mg sodium azide (storage RT)	**(Thermo Fisher Scientific, Inc.)**
Ad 500ml PBS (pH 7,2) (storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
EDTA 0.5 M (storage RT)	**(Applichem GmbH)**
Fixable Viability Dye eFluor® (storage -20°C)	**(Thermo Fisher Scientific, Inc.)**
Unsterile 96 well round bottom plate	**(Sarstedt AG & Co. KG)**
Eppendorf Centrifuge 5810R	**(Eppendorf AG)**
FACS tubes	**(Sarstedt AG & Co. KG)**
FACSCanto II	**(Becton Dickinson)**

All steps were performed on ice. A total of $1x10^6$ cells per gut section were used for Flow cytometric analysis. Antibody solutions were prepared in Fluopuffer according to the individual instructions. For multiple stainings, the antibodies were prepared in a solution.

Immediately after preparation, cells were transferred to round bottom plates and pelleted (1min, 700xg, 4°C). The supernatant was discarded and cells were resuspended in the Fixable viability Dye solution (100µl) to determine viable cells. After incubation (20min) on ice protected from light, 200µl of Fluo-buffer was added to stop the binding reaction. Subsequently, cells were pelleted (1min, 700xg, 4°C) and the supernatant was discarded by flipping the plate. Cells were resuspended in the respective primary antibody solution (50µl) and again incubated for 20min on ice protected from light. Adding 200µl Fluo-buffer, cells were washed (1min, 700xg, 4°C), resuspended in the secondary antibody solution and incubated for 20min stored on ice protected from light. At the end of the staining procedure, cells were resuspended in 120µl Fluo-buffer and transferred to a measuring tube. Before, the measuring tube had been prepared with 80µl of EDTA 1% (prepared in Fluo-buffer) and 200µl Fluo-buffer. Analyses were performed with a FACSCanto II (Becton Dickinson). BD FACS-DIVA Version 3.0 and FlowJo 10.8.1 were used for data analysis. Autofluorescence was corrected as described by Doyle et al. (376).

List of antibodies used for cell staining:

Antigen	Clone/Isotype/use	Concentration [µg/ml]	Secondary Ab	Concentration
chCD45	16-6 / IgG2a (purified)	2,5	Anti-mouse IgG2a (FITC) (Southern Biotech)	1:100
chMQ	KUL01 / IgG1 (Southern Biotech)	2	Anti-mouse IgG1 (APC) (Jackson Immuno Research)	1:1000
Haptene	Isotype control F71D7 (purified)	5		
chTCR γδ	TCR1 / IgG1/compensation control (purified)	5	Anti-mouse IgG1 (APC) (Jackson Immuno Research)	1:1000

Table 1.: Antibodies and staining conditions

For cell surface staining, resident gut macrophages were stained with anti-CD45, KUL01 mAbs or respective control mAbs (Isotype ctrl.) as primary mAbs. The cells were then identified via secondary Abs (APC/FITC labeled) according to standard procedures. Viability was assessed using fixable viability dye (APC-Cy7).

4.5.4 Culturing

Material (sterile)

RPMI 8/2/1 (37°C)	**(described under 4.3)**
96 well flat bottom plate	**(Thermo Fisher Scientific, Inc.)**
48 well flat bottom plate	**(Thermo Fisher Scientific, Inc.)**
24 well flat bottom plate	**(Thermo Fisher Scientific, Inc.)**
DPBS (pH 7,2, stored at 4°C)	**(Thermo Fisher Scientific, Inc.)**

4.5.4.1 Monocyte derived macrophages / splenic macrophages / bone marrow derived macrophages

After preparation, cells derived from blood spleen and bone marrow were resuspended in RPMI 8/2/1 and seeded in 24 well (1.5ml final volume per well), 96 well (200µl final volume per well) or 48 well plates (spleen cells) (1ml final volume per well) dependent on the experiment.

For optimal results, plates were prewarmed (40°C) and wells were filled with half of the final volume of warm RPMI 8/2/1 (37°C) prior to seeding to achieve an even distribution of cells in the wells. The plates were kept at 40°C and 5% CO_2 for a total of 72h. After 48h wells were carefully washed with sterile DPBS (37°C). A second washing step was performed with DPBS

(300µl in 96 well plate; 2ml in 24 well plate and 1ml in 48 well plate). Subsequently, fresh RPMI 8/2/1 (100µl) was added and cells were further cultivated at 40°C and 5% CO_2 for an additional 24h.

4.5.4.2 Intestinal macrophages (ileum / cecum)

Following preparation, cells were seeded in prewarmed 96 well plates prefilled with 100µl/well of RPMI 8/2/1 (37°C) and incubated at 40°C 5% CO_2 for 24h. Half of the supernantant was replaced by fresh cell culture media every 24h until stimulation with SCFAs and LPS 72h after seeding.

4.5.5 Stimulation-kinetics (butyrate)

Material (sterile):

24 well flat bottom plates	**(Thermo Fisher Scientific, Inc.)**
Sodium butyrate 200mM (37°C)	**(described under 4.2)**
LPS (Salmonella Typhimurium LPS) (storage 4°C)	**(Sigma Aldrich GmbH)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**

Following isolation (see 4.5.2) cells derived from monocytes, spleen and bone marrow were seeded in 24 well plates (cell counts: $1.8x10^7$ cells/well monocyte derived macrophages; $3x 10^7$/well splenic macrophages; $1.9x10^7$/well bone marrow) and incubated as described under 4.5.4. A 2mM sodium butyrate solution was prepared in prewarmed RPMI 8/2/1. The cell culture supernatant was removed and the 2mM butyrate solution (37°C) was added (500µl per well final volume) into the respective wells. Cells were incubated at 40°C and 5% CO_2 for 24h. LPS was diluted to a concentration of 100ng/mL in the respective 2mM sodium butyrate solution (37°C). The cell culture supernatant was discarded and the LPS was applied in the presence of butyrate 2mM in fresh RPMI 8/2/1 (400µl final volume per well; 37°C). Control wells were either treated with butyrate 2mM alone or with RPMI 8/2/1 (37°C) in the absence of butyrate. Cells were incubated at 40°C in a 5% CO_2-environment. Griess assay was used as described under 4.1 to measure NO-production after 4h, 6h, 8h, 24h and 48h of LPS stimulation. All assays were performed in technical triplicates. Three animals were used in this assay.

4.5.6 Stimulation- SCFAs

4.5.6.1 Monocyte derived macrophages / splenic macrophages / bone marrow derived macrophages

Material (sterile):

96 well flat bottom plates	**(Thermo Fisher Scientific, Inc.)**
Sodium butyrate 200mM (37°C)	**(described under 4.2)**
Sodium propionate 200mM (37°C)	**(described under 4.2)**
Sodium acetate 200mM (37°C)	**(described under 4.2)**
LPS (S. Typhimurium LPS) (37°C)	**(Sigma Aldrich GmbH)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**

Following isolation (see 4.5.2) cells derived from monocytes, spleen and bone marrow were seeded in 96 well plates (cell counts: $1.8x10^7$ cells/well monocyte derived macrophages; $3x\ 10^7$/well splenic macrophages; $1.9x10^7$/well bone marrow) and incubated as described under 4.5.4.1. SCFAs solutions were prepared in prewarmed RPMI 8/2/1 at different concentrations (4mM and 2mM for propionate and acetate, 0.5mM/1mM/2mM/4mM for butyrate). The cell culture supernatant was removed and the SCFAs solutions were applied in the respective wells (100µl per well final volume).

In two other experiments in which butyrate was added at later time-points, the wells were supplied with fresh medium instead of adding butyrate. Cells were further incubated at 40°C and 5% CO_2 for 24h.

LPS was adjusted to a concentration of 100ng/mL in the respective SCFAs solutions. The cell culture supernatant was removed and LPS was applied to the cells in the presence or absence of SCFAs (100µl final volume per well).

In this way, in one experimental approach, butyrate 2mM and LPS were simultaneously applied to the wells without prior incubation with butyrate.

In addition, in another approach, this way, the cells were no longer treated with butyrate 2mM, but only with LPS. Thus, butyrate was incubated for 24h and then removed from the cells, by adding LPS solution in the absence of butyrate. Cells were further incubated at 40°C and 5% CO_2 for 24h.

4h after LPS treatment, another experimental approach was tested. Therefore, butyrate 2mM was added to the corresponding wells that had been pretreated exclusively with LPS. Thus, 1µl of the cell culture supernatant was removed and 1µl of a 200mM butyrate solution was added to the corresponding wells. The cells were then further incubated at 40°C and 5% CO_2.

Control wells were treated with or without the corresponding SCFA solutions (2mM and 4mM) in the absence of LPS (100µl final volume per well). Griess assay was used as described under 4.1 to measure NO production after 24h of LPS stimulation.

All assays were performed in technical triplicates. Three animals were used in the case of monocyte derived macrophages and cells derived from spleen. Bone marrow derived cells were prepared from four animals.

4.5.6.2 Intestinal macrophages (ileum / cecum)

Material (sterile):

96 well flat bottom plates	**(Thermo Fisher Scientific, Inc.)**
Sodium butyrate 200mM (37°C)	**(described under 4.2)**
LPS (Salmonella Typhimurium LPS) (37°C)	**(Sigma Aldrich GmbH)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**

Following isolation (see 4.5.2.4), cells derived from ileum ($2x10^6$ cells/well) and cecum ($1.5x10^6$ cells per well) were seeded in 96 well plates and incubated as described under 4.5.4.2. A sodium- butyrate solution (2mM) was prepared as described under 4.5.6.1. The cell culture supernatant was removed and butyrate 2mM was applied in the respective wells (100µl per well final volume). The cells were then further incubated for 24h at 40°C and 5% CO_2.

LPS was adjusted to a concentration of 100ng/mL in a butyrate 2mM solution.

The cell culture supernatant was removed and the LPS-butyrate solution was applied to the cells (100µl final volume). Cells were further incubated at 40°C and 5% CO_2. All assays were performed in technical triplicates. Four independent replicates were done.

4.5.7 Re-stimulation of primary cells

4.5.7.1 Culturing of the cells

Material (sterile):

96 well flat bottom plates	**(Thermo Fisher Scientific, Inc.)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**

In a follow-up experiment, all primary cells derived from blood, spleen, bone marrow, ileum and cecum were further cultured. After initial stimulation (described under 4.5.6) the wells were replenished with RPMI 8/2/1 and cells were further incubated at 40°C and 5% CO_2 for 24h.

The supernatant was then completely discarded and the wells were replenished with fresh RPMI 8/2/1 (37°C).

Cells were then further incubated at 40°C and 5% CO_2. In the following 7 to ten days, the supernatant was renewed every second day.

4.5.7.2 Intermediate control

Material (sterile):

96 well flat bottom plates	**(Thermo Fisher Scientific, Inc.)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**

Since some of the cells had been stimulated with LPS, the cell culture supernatant was examined for NO production prior to the second application of LPS (described under 4.5.7.3).

NO production was measured using Griess assay as described under 4.1.

4.5.7.3 Re-stimulation

Material (sterile):

96 well flat bottom plates	**(Thermo Fisher Scientific, Inc.)**
LPS (Salmonella Typhimurium LPS) (37°C)	**(Sigma Aldrich GmbH)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**

LPS was diluted in RPMI 8/2/1 at a final concentration of 100ng/ml.

Immediately after measurement of the intermediate control (described under 4.5.7.2), the remaining cell culture supernatant was removed and LPS (100ng/ml) was applied to the cells (100µl final volume per well). In this experiment, the respective controls from section 4.4.6 were additionally treated with LPS. Cells were incubated at 40°C and 5%CO_2 for 24h. Subsequently, NO levels were measured as described under 4.1.

4.6 RNA preparation

4.6.1 Culturing of splenic cells for RNA preparation

Material (sterile):

96 well flat bottom plates	**(Thermo Fisher Scientific, Inc.)**
Sodium butyrate 200mM (37°C)	**(described under 4.2)**
LPS (S. Typhimurium LPS) (37°C)	**(Sigma Aldrich GmbH)**
RPMI 8/2/1 (37°C)	**(described under 4.3)**
DPBS (pH 7,2, stored at 4°C)	**(Thermo Fisher Scientific, Inc.)**

Macrophages derived from spleen were isolated as described under 4.5.2.2. and cultured in 48 well plates as described under 4.5.4.1 ($3x10^7$ cells per well).

Cells were pretreated with or without butyrate 2mM for 24h (500µl/well) as described under 4.5.6.1 and subsequently cultured with or without LPS in the presence or absence of butyrate (500µl final volume per well). After 8h and 24h of LPS stimulation, cells were washed thoroughly with DPBS and RNA was isolated as described under 4.6.2. Depending on the cell yield level of the wells after washing, 3 to 6 wells were used for RNA preparation.

4.6.2 RNA isolation

All steps were performed under aseptic conditions (PCR Workstation, Peqlab Biotechnologie GmbH)

Material:

ReliaPrep RNA Cell Miniprep isolation system	**(Promega GmbH)**
BIO Vortex V1	**(Peqlab Biotechnologie GmbH)**
Eppendorf Centrifuge 5425R	**(Eppendorf AG)**
Isopropanol (storage RT)	**(Omnilab-Laborzentrum GmbH & Co. KG)**
DPBS (pH 7,2, storage 4°C)	**(Thermo Fisher Scientific, Inc.)**
RNAse free cups	**(Sarstedt AG &Co. KG)**

The ReliaPrep RNA Cell Miniprep isolation system was used to prepare total RNA from macrophage preparations. Cells were cultured as described under 4.5.4.1 and subsequently thoroughly washed with 250µl sterile PBS (4°C). A cell lysing solution was prepared by mixing 60µl of TG buffer (stored at 4°C) with 6ml BL buffer. 600µl of the lysing solution was pipetted into each well of the cell culture plate and incubated for 3 minutes. Next, the cell pellet was dispersed by resuspending the lysing solution several times (approx. 10 times) and then transferred to an RNAse-free cup. An equal amount of Isopropanol was added and the cell lysate was mixed with Ispropanol by vortexing five seconds. Per sample two collection tubes, one minicolumn and one elution tube were unpacked and labeled. The minicolumn was placed in one elution tube and the cell lysate was transferred to the minicolumn (500µl). To bind the RNA to the minicolumn, the lysat was centrifuged at 14000xg for 30 seconds at RT. 500µl of RNA Wash Solution was added to the minicolumn. Subsequently the minicolumn was centrifuged at 14000xg for 30 seconds. The collection tube was emptied and a DNAse I incubation mix containing Yellow Core Buffer, $MnCl_2$ and DNAse I was prepared according to the amounts in table 2. 30µl of the DNAse I incubation mix were added to the membrane of each minicolumn and the samples were incubated at RT for 45 minutes. Subsequently, 200µl of Column Wash Solution was added to the minicolumn and centrifuged at 14000xg (RT) for 30 seconds. 500µl of RNA Wash Solution was added and the samples were centrifuged at 14000xg for 30 seconds. The collection tube was discarded and the minicolumn was placed into a new collection tube. 300µl of RNA Wash solution was added to the minicolumn and centrifuged at high speed for 2 minutes. Subsequently the minicolumn was transferred to an elution tube and 25µl of nuclease free water was added to the minicolumn. The nuclease free water was incubated at RT for 10min to dissolve the RNA from the membrane of the

minicolumn. Subsequently, the elutiontube was centrifuged at 14000xg for 1 minute with the lid facing to the outside. The minicolumn was discarded and the RNA was immediately stored on ice. RNA quality control and quantification was performed using the Nanodrop 1000 instrument as described under 4.5.3. Subsequently, the samples were stored at -80°C.

Solution	**Volume per sample [µl]**
Yellow Core Buffer	24
$MnCl_2$	3
DNAse I	3

Table 2.: DNAse I incubation mix

4.6.3 Contamination control by Nanodrop 1000

The occurrence of potential contaminants such as proteins or chaotropic salts was measured using the NanoDrop 1000 instrument. As blank 1µl of nuclease-free water was used prior to sample testing. 1µl of each RNA sample was applied subsequently to evaluate RNA purity. The 260/280 and 260/230 ratios were assessed. RNA was considered uncontaminated if both ratios exceeded 1.8. Samples representing lower values were precipitated as described under 4.6.4.

4.6.4 Clearance of RNA contaminations

Material:

BIO Vortex V1	**(Peqlab Biotechnologie GmbH)**
Eppendorf Centrifuge 5425R	**(Eppendorf AG)**
Isopropanol (storage RT)	**(Omnilab-Laborzentrum GmbH & Co. KG)**
RNAse free cups	**(Sarstedt AG &Co. KG)**
Sodium acetate 3M (storage RT)	**(Thermo Fisher Scientific, Inc.)**
Ethanol 75% (storage RT)	**(Omnilab-Laborzentrum GmbH & Co. KG)**
Nuclease-free Water (storage 4°C)	**(Promega GmbH)**

Samples were thawed on ice. An equal volume of isopropanol and 1/10 volume of sodium acetate 3M was added to the contaminated RNA solution. The solution was gently mixed and incubated at RT for 10min. Samples were centrifuged at 12.000xg for 10min at 6°C, the

supernatant was discarded and 1ml of ethanol (EtOH) 75% was added to each sample. Next, samples were centrifuged at 7.600xg for 5min at 6°C and the supernatant was discarded. To remove residual EtOH, samples were again shortly centrifuged and the supernatant was removed using a pipette. To evaporate the EtOH, the pellet was dried with opened caps at RT for approx. 30min. The pellet was dissolved in 20µl nuclease-free water and purity was determined with the NanoDrop 1000 instrument (see. 4.6.3).

4.6.5 Quality control by Agilent 2100 Bioanalyzer

All steps were performed under aseptic conditions (PCR Workstation, Peqlab Biotechnologie GmbH)

Material:

Isolated RNA	
Eppendorf Centrifuge 5425R	**(Eppendorf AG)**
RNA 6000 Nano Kit (storage 4°C)	**(Agilent Technologies GmbH)**
Agilent 2100 Bioanalyzer	**(Agilent Technologies GmbH)**
BIO Vortex V1	**(Peqlab Biotechnologie GmbH)**
Thermomixer comfort	**(Eppendorf AG)**
Nuclease-free water (storage 4°C)	**(Promega GmbH)**
RNAse ZAP (storage RT)	**(Sigma Aldrich GmbH)**

All reagents were used at RT. Prior to use a heating block was prepared (70°C). A spin filter was used to prepare 550µl of RNA Nano Gelmatrix (10min; 1.500xg; RT). Subsequently, 65µl of filtered RNA Nano Gelmatrix was transferred to a nuclease-free 1.5ml tube. After adding 1µl of dye the gel-dye mix was centrifuged for 10min at 13,000xg. RNA samples were thawed on ice, incubated for 2min at 70°C and immediately put back on ice. The Bioanalyzer electrodes were cleaned using 350µl of RNAse ZAP pipetted into a cleaning chip (1min). Likewise, afterwards a separate cleaning chip was used with nuclease-free water two times. Subsequently, electrodes were dried with the device open for one minute. The priming station was set to position C and a new RNA Nano Chip was added to the priming station. 9µl of filtered gel-dye mix was pipetted to the well with a white G on black ground and the priming station was closed for exactly 30s (seconds). The station was opened and 9µl of gel-dye mix was added to the two wells with a black G on bright ground. Each sample well and the well with the ladder symbol was filled with 5ul of RNA 6000 Nano marker. 1µl of each sample or 1µl of Nano ladder was added to the respective wells. The chip was vortexed in a Vortex mixer for 1min and transferred to the Bioanalyzer.

The program “Agilent 2100 Expert” was initiated, the samples were labeled and the Bioanalyzer run was started.

The Agilent 2100 Bioanalyzer system uses a chip based capillary electrophoresis to evaluate the quality of RNA samples. RNA molecules were separated by size. Based on the electropherogram and the 18S and 28S rRNA peaks, the software calculates the RNA integrity number (RIN). RNA integrity can be lowered by RNAses, heat, contaminations with DNA or shear forces.

The RIN cutoff for qPCR experiments was set to 8.

4.7 Quantitative real-time polymerase chain reaction

All steps were performed under aseptic conditions (PCR Workstation, Peqlab Biotechnologie GmbH)

4.7.1 cDNA synthesis

Material:

DNase digested RNA (storage -80°C)
GoScript™ Reverse Transcription Mix,
Random Primer System (storage -20°C) (Promega GmbH)
Nuclease-free water (storage 4°C) (Promega GmbH)
MJ Mini Personal Cycler (Bio-Rad Laboratories, Inc.)

GoScript reaction buffer Random Primer was thawed on ice and GO Script Enzyme Mix was kept at -20°C during the whole process. Both reagents were mixed gently and briefly centrifuged. The required amount [µl] of 400ng RNA was calculated based on the quantitative Nanodrop measurements. Nuclease free water was added to a final volume of 10µl. The Thermal cycler was prewarmed to 25°C. The required amounts of GOScript Reaction buffer and Enzyme Mix were subsequently added to the nuclease free water in a sterile cup.

A mastermix was prepared on ice according to the amounts in table 3.

Reagent	**Amount for one sample [µl]**
Nuclease-free water	4
GO Script Reaction buffer Random Primer	4
GO Script Enzyme Mix	2
Total	**10**

Table 3: Reagents used in cDNA synthesis

The mastermix was added to 10µl of the RNA dilution and subsequently placed in a programmed thermocycler.

Thermal cycler adjustments:

5min at 25°C

60min at 42°C

15min at 70°C

Immediately after the thermal cycler run, samples were put on ice and incubated for at least 5min. The cDNA was used immediately or stored at -20°C.

4.7.2 Quantitative real-time polymerase chain reaction

Material:

cDNA (storage -20°C)
GoTaq qPCR Master Mix **(Promega GmbH)**
Nuclease-free water (storage 4°C) **(Promega GmbH)**
qPCR Primer (storage -20°C) **(Erurofins Genomics GmbH)**
7500 FAST Real-Time PCR System **(Thermo Fisher Scientific, Inc.)**
96-well qPCR plate **(Sarstedt AG &Co. KG)**
Sealing film for qPCR plates **(Sarstedt AG &Co. KG)**

Quantitative results of expression levels of a requested gene can be obtained using the quantitative real-time polymerase chain reaction. Primers and respective sequences can be found in table 2. RPL-13 was used as a housekeeping gene. cDNA was diluted 1:20 with nuclease-free water to obtain a final concentration of 20ng/µl.

Gene	Primer Sequence 5´- 3´	NCBI Accesion number
RPL-13	Forward - GAGGTGCCCGACTGTCAGAT Reverse - ATCGTCCGAGCAAACCTTTTGT	NM_204999.1
IL-1ß	Forward - CTGAGTCATGCATCGTTTATGTTTC Reverse - AAATACCTCCACCCCGACAAG	NM_204524
IL-6	Forward - GCTTCGACGAGGAGAAATGC Reverse - GCCAGGTGCTTTGTGCTGTA	NM_204628
IL-8	Forward - CTGGCCCTCCTCCTGGTTTC Reverse - TGGCGTCAGCTTCACATCTTG	NM_205498
iNOs	Forward - AAGCAAACGGCCAAGATCCA Reverse - CCCACCTCAAGGAGCATGTTG	NM_204961
TNF-α	Forward - CGCTCAGAACGACGTCAA Reverse - GTCGTCCCACACCAACGAG	MF000729

Table 4: qPCR primers used in quantitative real-time PCR

A mastermix was prepared according to the amounts in table 5.

Reagent	Amount for 1 sample [µl]
Primer sense	1.5
Primer antisense	1.5
Nucl.- free water	4.5
2x GoTaq Mastermix	12.5
cDNA	5
Total	25

Table 5: Reagents for preparing a Mastermix for qPCR reaction

All components were stored on ice protected from light. All pipetting steps were performed at 4°C. Components 1-4 were mixed and 20µl was distributed on the 96-well plate. 5µl per sample of diluted cDNA was added to each well in duplicates. The plate was sealed with a sealing film and shorty spinned down to remove air bubbles. The qPCR cycler was programmed as follows:

Step	Function	T	No of cycles	time
Initial activation		95°C	1x	2min
Amplification	Denaturation	95°C	40x	15s
	Annealing	59°C		30s
	Extension	72°C		30s
Melting curve		95°C	1x	15s
		57°C		30s
		95°C		15s

Table 6: Settings of the quantitative real-time PCR for amplification of immunorelevant genes

To generate reproducible results in the qPCR, the cycle threshold (CT) was determined in the logarithmic phase. Means of the duplicates were calculated and CT values were normalized against the housekeeping gene by subtracting the CT value of the RPL-13 rRNA from the CT value of the target gene:

$$\Delta CT = CT_{(target\ gene)} - CT_{(housekeeping\ gene)}$$

To obtain directly proportional results, the ΔCT was further subtracted from the total amount of amplification cycles (40). Ct values exceeding the Ct- standardkurve were set to 30.

5 RESULTS

This study aimed to investigate the effects of SCFAs on chicken macrophages derived from different tissue sources.

First, the chicken macrophage cell line HD11 and the murine macrophage cell line RAW 264.7 were used as a model system. The effect of SCFAs was examined comparing NO content in the cell culture supernatant following LPS treatment.

Second, primary macrophages were isolated from the blood, spleen, bone marrow, ileum and cecum. In three dependent follow-up experiments the effects of SCFAs were investigated under different conditions.

Third, cytokine expression levels were examined in macrophages derived from spleen following butyrate and LPS treatment.

5.1 NO-Assay-cell line experiments

5.1.1 HD11 cell line

HD11 cells have been shown to produce nitric oxide in response to LPS. To determine an appropriate LPS concentration for subsequent experiments, bacterial derived LPS was applied in a log2 scale and NO production was quantified by measuring the degradation product of NO. After 24h, NO_2- measurement revealed that HD11 cells which were exclusively treated with different concentrations of LPS react with a concentration-dependent production of NO (figure 1).

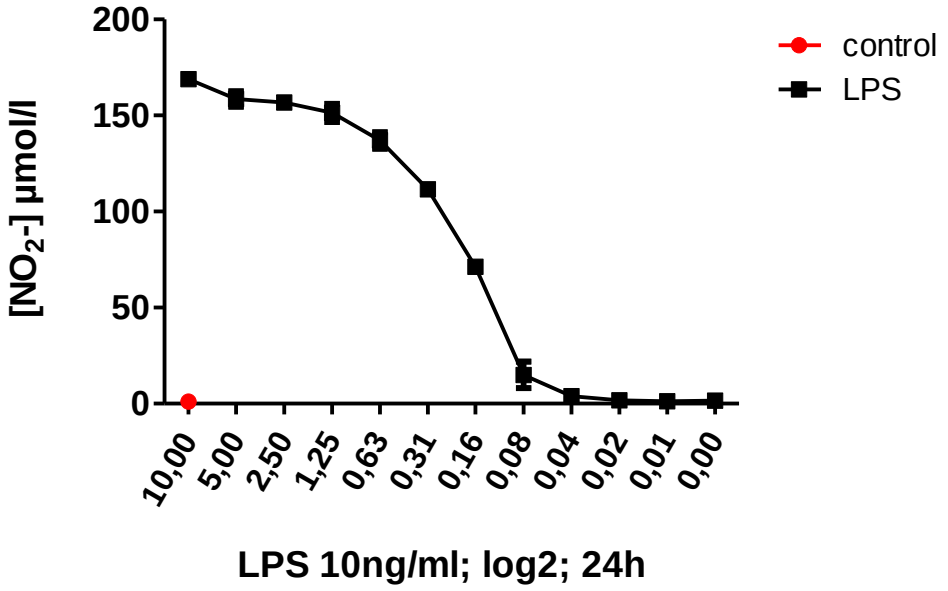

Figure 1: Dose response of HD11 cells to LPS treatment; n=1; 3 replicates

Chicken HD11 cells produce nitric oxide in response to LPS in a dose dependent manner.

To determine a suitable time point for subsequent experiments, two different LPS concentrations were used and the supernatant was tested for NO production at different time points. Over a period of 24h NO levels increased continuously. After 12h-24h a dose and time response relationship was most evident at a LPS concentration of 5ng/ml (figure 2).

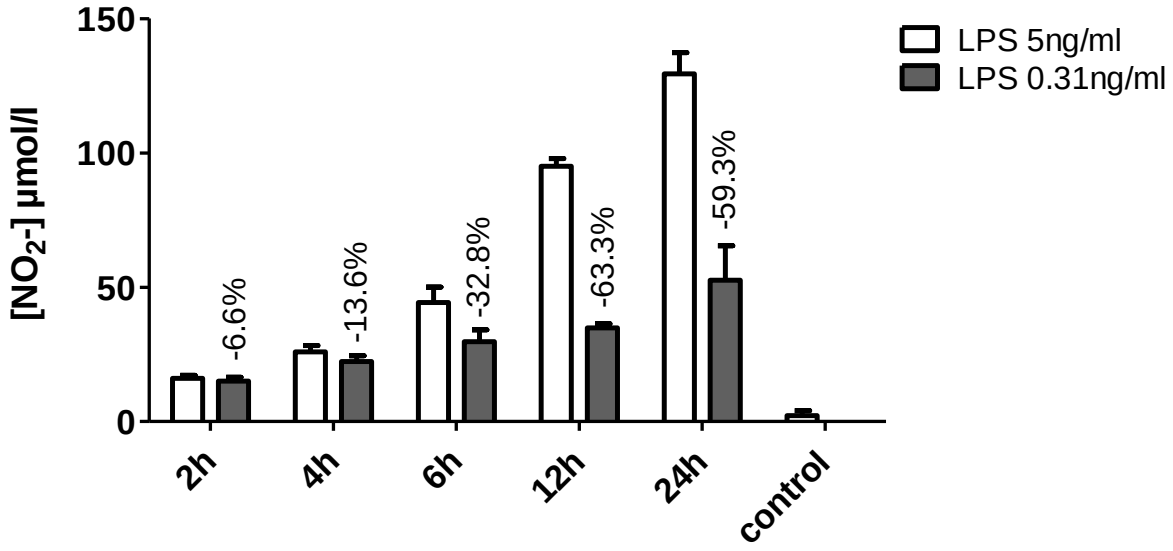

Figure 2: Dose and time response of HD11 cells due to LPS treatment; n=1; 3 replicates

The nitric oxide production of LPS stimulated HD11 increases over a time period of 24h in a dose dependent manner. Percent decrease values were calculated against LPS 5ng/ml.

After 48h of LPS treatment detachment of cells and shrinkage was observed. Thus, the 24h time point was used in subsequent experiments.

Next, we hypothesized that SCFAs treatment exerts anti-inflammatory effects on chicken HD11 cells. To test this hypothesis, this experiment was modified and SCFAs were added for 24h prior to additional LPS treatment. Different LPS concentrations were used. Cells treated with butyrate and LPS displayed a significantly lower NO production in comparison, which could also be seen to a lesser extend in propionate treated and LPS stimulated cells. Acetate only had an inhibitory effect at higher LPS concentrations as revealed by percent decrease values (figure 3).

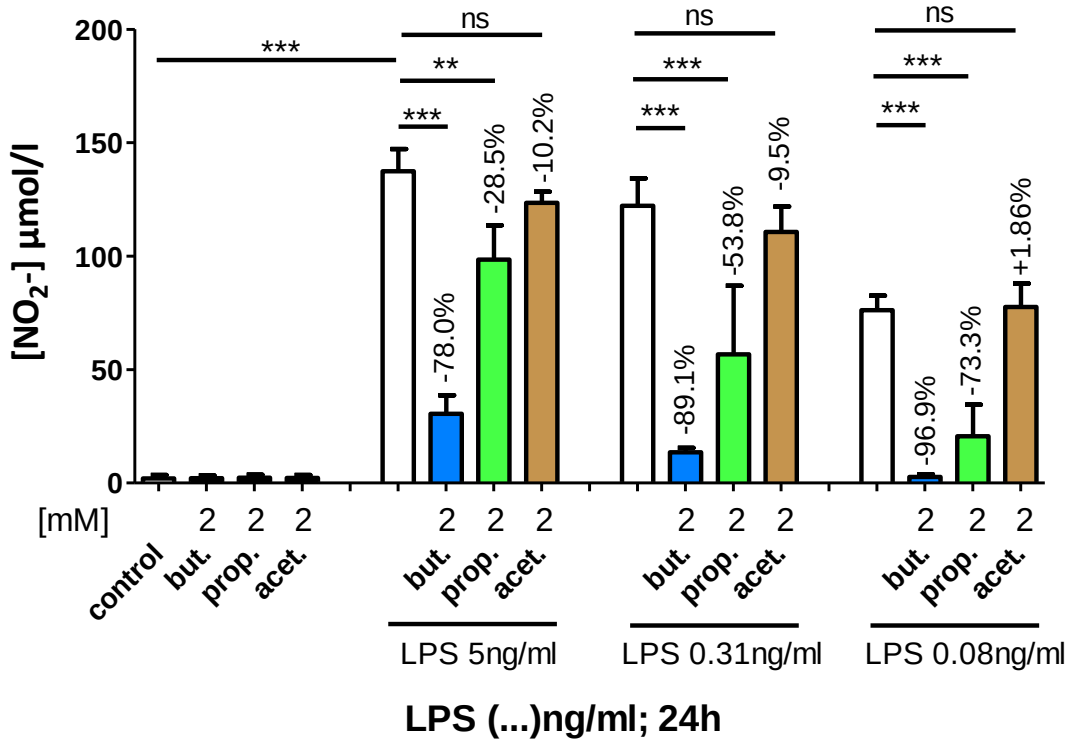

Figure 3: Impact of SCFA treatment on HD11 cells at different LPS concentrations; n=3 independent experiments with 3 replicates per experiment

NO values are represented as means with standard deviation. Butyrate and to a lesser extend propionate significantly reduced the NO production of LPS stimulated HD11 cells. Statistics were performed using two-way ANOVA. One asterisk indicates a p-value< 0.05, two asterisks indicate a p-value< 0.01, three asterisks indicate a p-value< 0.001. Percent decrease values were calculated against the respective LPS values. but.= butyrate; prop.= propionate; acet.= acetate

5.1.2 RAW 264.7 cell line

To compare our data with the findings described in the mammalian literature, butyrate was tested on murine RAW 264.7 cells. Like HD11 cells, RAW 264.7 cells responded with the production of NO upon LPS stimulation in a dose dependent manner. Treatment with butyrate resulted in a reduction of nitric oxide levels. A dose response relationship was still evident in butyrate treated cells (figure 4).

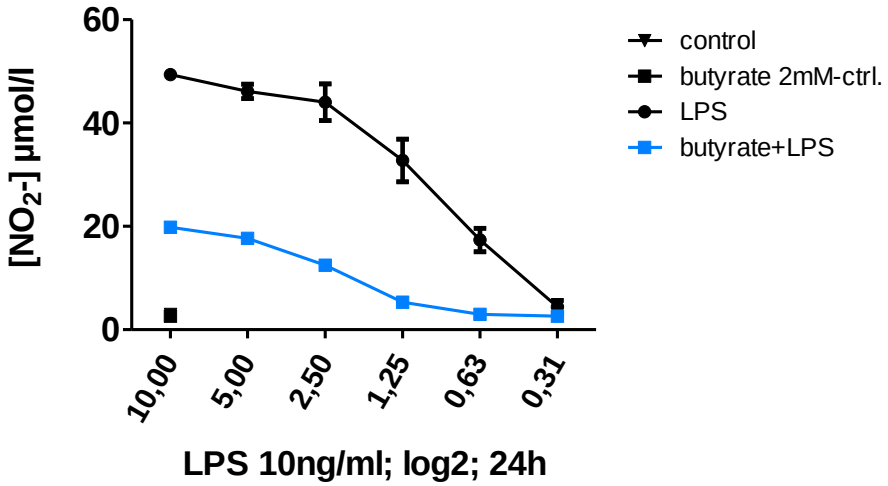

Figure 4.: Impact of butyrate treatment on RAW 264.7 cells; n=1; 3 replicates

The murine macrophage cell line RAW 264.7 produces NO in response to LPS. This effect is less evident in butyrate treated cells. Data represent means and standard deviations.

5.2 NO-Assay- primary cells

The experiments with the cell lines described above showed a clear influence of SCFAs on NO production of LPS-stimulated cells. To get closer to the situation in the living animal the effects of SCFAs were also examined on primary macrophages.

5.2.1 Stimulation of monocyte derived macrophages / splenic macrophages / bone marrow derived macrophages

To determine a suitable time point for subsequent experiments LPS was added to primary monocyte-derived, bone marrow derived and splenic macrophages and the cell culture supernatant was examined for NO production after 4h, 6h, 8h, 24h and 48h using the Griess assay. Cells treated with LPS alone produced significant amounts of NO after just 4h and an increase of NO production was observed over a period of 48h. Spearman rho calculations revealed significant time to NO correlations in every cell culture system stimulated with LPS.

At every time point measured NO levels in butyrate treated cells were lower than in the respective cells solely treated with LPS as represented by percent decrease values. These findings indicate that butyrate exerts anti-inflammatory effects on primary macrophages regardless of the tissue origin (figure 5).

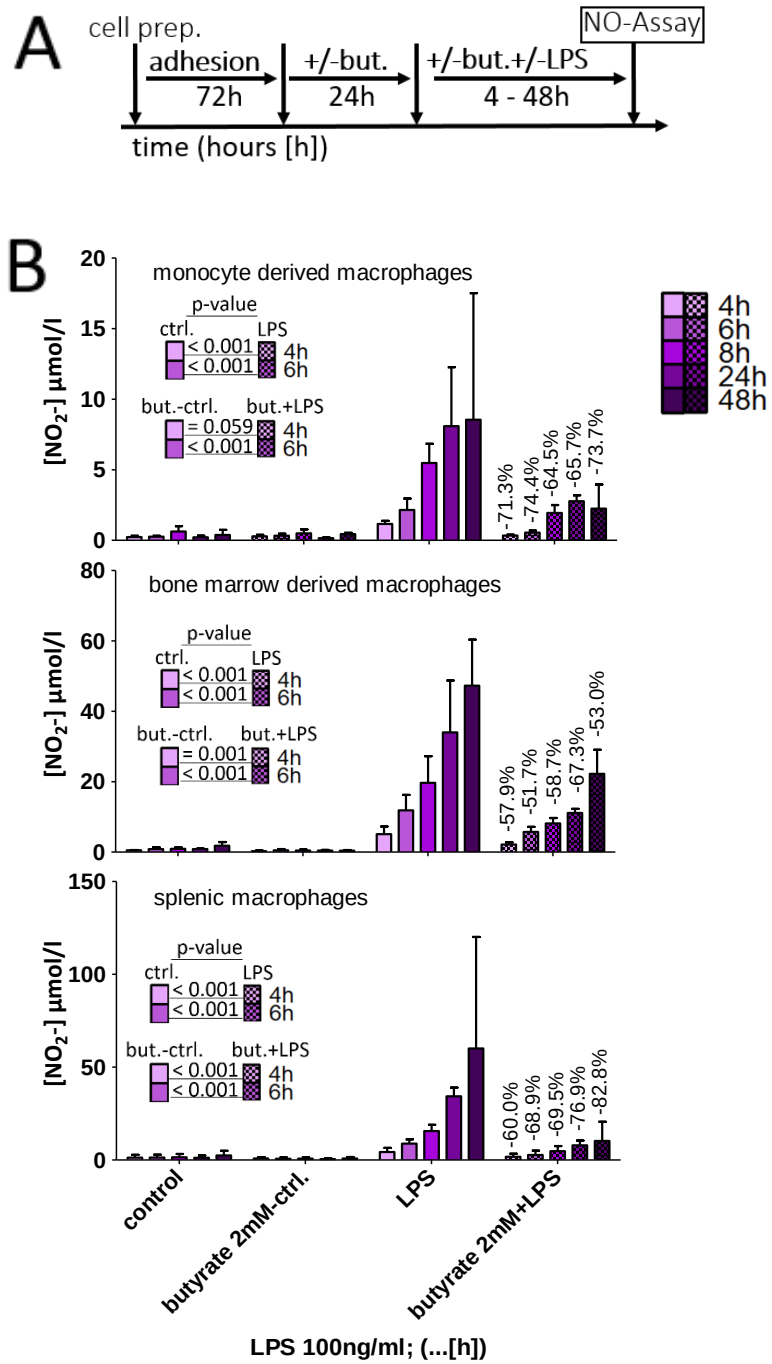

Figure 5: Kinetics of butyrate dependent and independent responses to LPS treatment in primary macrophages; n=3; 3 replicates

A) Treatment scheme B) NO levels increase over a time period of 48h in LPS stimulated primary cells. Butyrate treated cells produced less NO in comparison. Data represent means and standard deviations. Percent decrease values were calculated within LPS-stimulated cells between butyrate-treated and non-butyrate-treated cells. Statistics were performed using Wald Chi square. Spearman rho calculations were significant in both LPS treated and butyrate and LPS treated cells ($p < 0.001$) in every organ system. but.= butyrate

In addition to butyrate, in another experimental approach the effects of propionate and acetate were tested at different concentrations in the absence or presence of LPS.

Butyrate and, to a lesser extent, propionate inhibited NO production by LPS-stimulated splenic macrophages as well as bone marrow and monocyte derived macrophages in a concentration dependent manner. In butyrate treated cells, spearman rho calculation revealed significant concentration dependent correlations in splenic ($p< 0.001$) and bone marrow derived cells ($p= 0.003$). Percent decrease values revealed a slight concentration dependent NO production in monocyte derived cells. Concentration dependent effects of propionate were most evident in monocyte derived and splenic cells. In acetate treated cells higher nitric oxide values were present at higher concentrations in monocyte and bone marrow derived cells (figure 6).

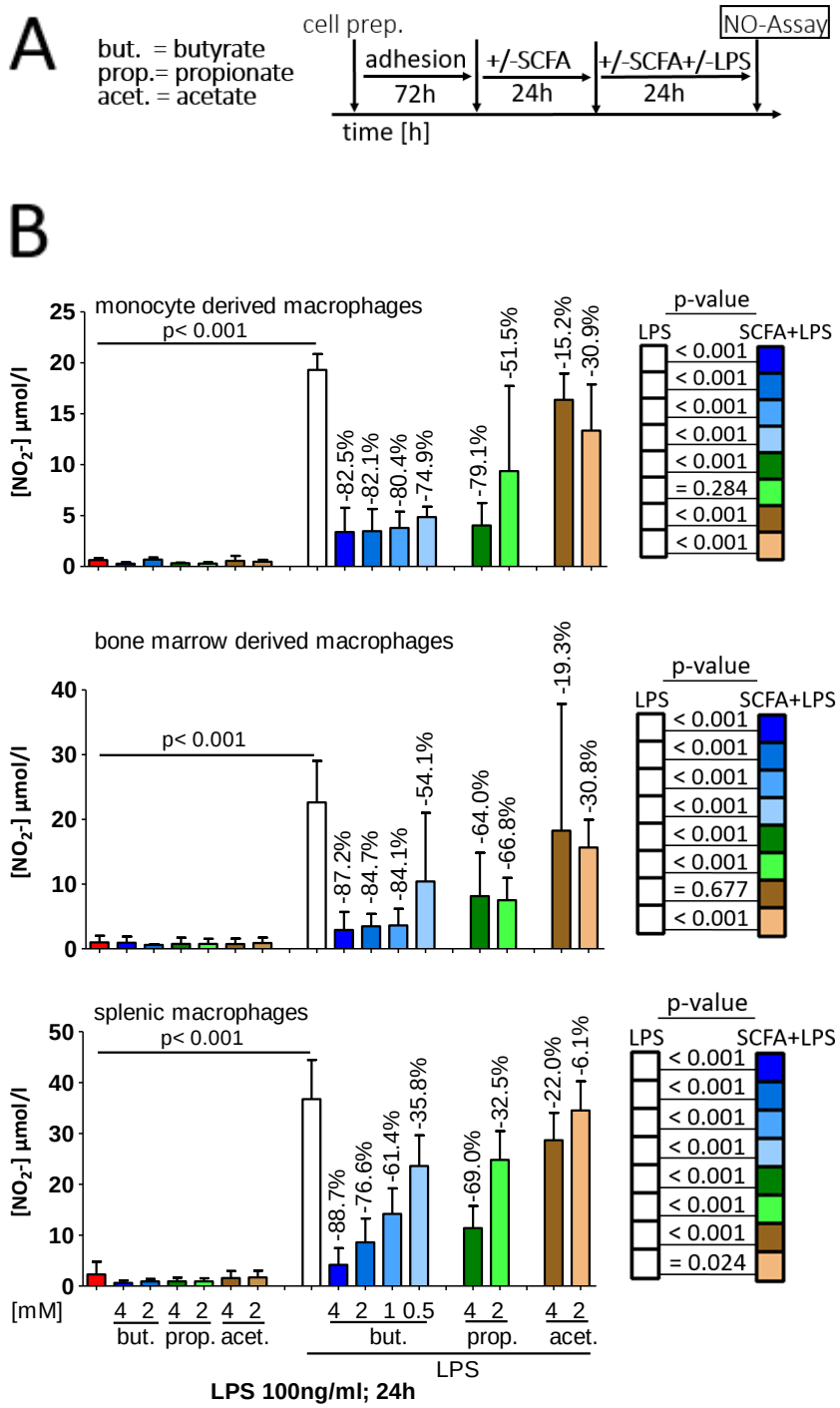

Figure 6: Dose response in SCFA treated primary cells to LPS treatment; n=3 in monocytes and macrophages derived from spleen; n=4 in bone marrow derived cells; 3 replicates

A) Experimental setup B) NO production in LPS treated primary cells in the absence or presence of SCFAs.

Nitric oxide levels were decreased in cells treated with SCFAs and LPS. Data represent means and standard deviations. Percent decrease values were calculated against LPS treated cells. Statistics were performed using Wald Chi square. Spearman rho calculations were significant in butyrate treated cells derived from spleen (p< 0.001) and bone marrow (p= 0.003)

Butyrate displayed the most suppressive effect on NO production. To further investigate the anti-inflammatory properties of butyrate the following additional experimental conditions were investigated:

1. The cells were pre-incubated for 24h with butyrate and subsequently stimulated with LPS in the absence of butyrate A
2. Butyrate and LPS were administered simultaneously B
3. After 4h of LPS treatment cells were additionally incubated with butyrate C

After 24h of LPS stimulation NO levels revealed that butyrate inhibited the NO release regardless of the experimental approach (figure 7).

Percent decrease values revealed that pre-treatment with butyrate exerts the most inhibiting effect on NO production in cells derived from spleen and bone marrow. This effect was less evident in monocyte derived cells.

In macrophages derived from spleen and bone marrow, simultaneous treatment with butyrate and LPS showed that these cells respond with significant NO production, which, however, was inhibited by the effect of butyrate. However, the butyrate-mediated inhibition was most prominently observed in blood cells in this experimental setting. The belated application of butyrate also influenced the NO production. The 4h time point was chosen in this experimental setup because NO values were detected just 4h after LPS stimulation (figure 5).

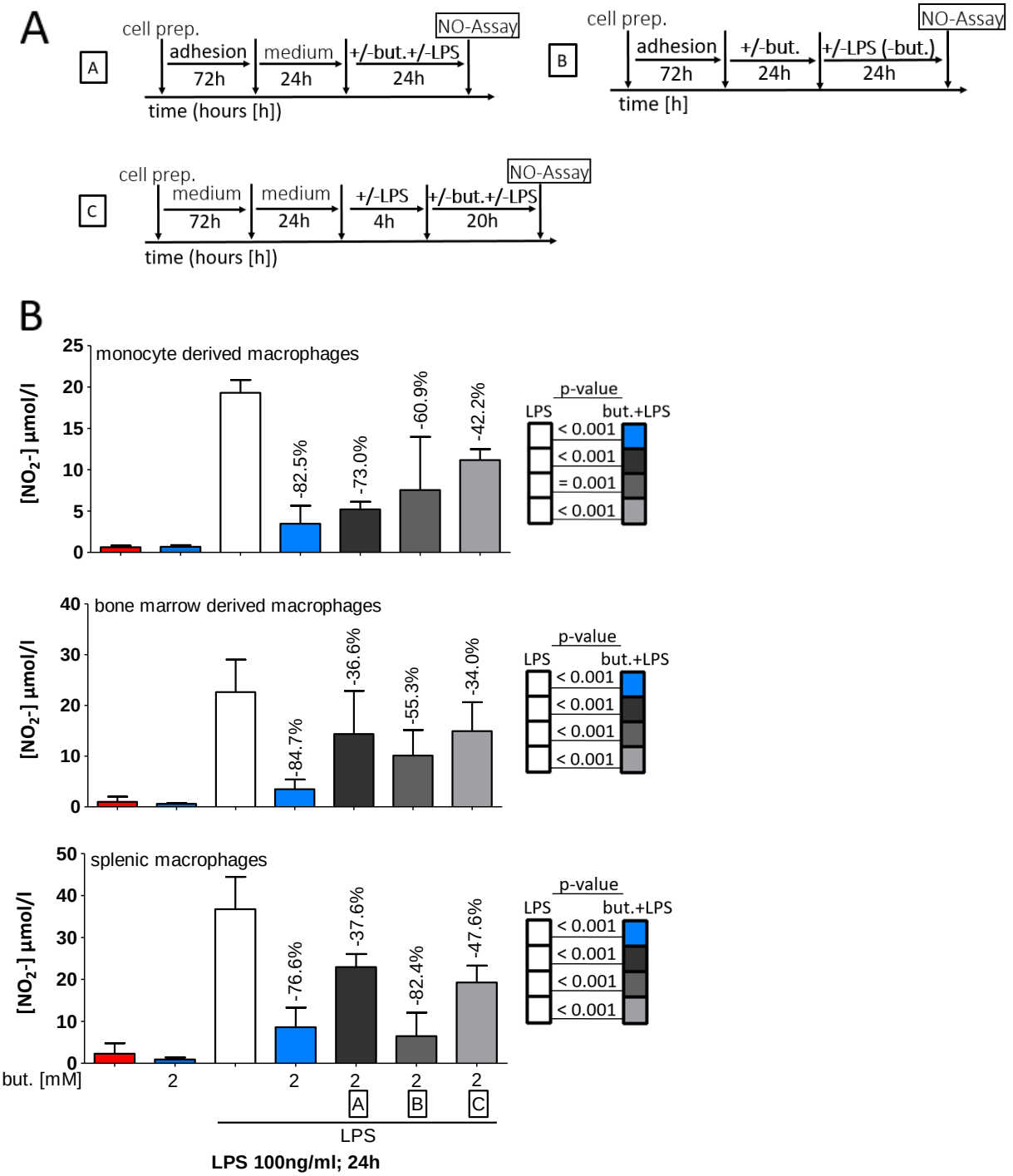

Figure 7: Application dependency of butyrate; n=3 in monocytes and macrophages derived from spleen; n=4 in bone marrow derived cells; 3 replicates

A) Experimental setup B) NO production in LPS treated primary cells in the absence or presence of butyrate.

The blue, white and red bars are extracted from figure 6. Nitric oxide levels were significantly decreased in cells treated with butyrate and LPS regardless of the experimental setup. Data represent means and standard deviations. Percent decrease values were calculated against LPS treated cells. Statistics were performed using Wald Chi square.

5.2.2 Stimulation of intestinal macrophages (ileum / cecum)

Macrophages in the gut are confronted with a plethora of foreign antigens. In mammals, intestinal macrophages display an inflammation anergic phenotype with reduced capacity of nitric oxide production. In chickens, information on intestinal macrophages is scarce. Therefore, cells were isolated from 8- to 9-week-old chickens and subsequently stimulated with LPS to test the effects of LPS and butyrate on chicken gut derived macrophages. Cells were isolated from ileum and cecum, identified using fluorescence assisted cell-sorting (figure 8) and cultured. Butyrate was applied for 24h followed by additional LPS stimulation. Upon stimulation with LPS, ileal as well as cecal macrophages responded with the production of NO. Treatment with butyrate reduced NO levels in both cases (figure 9).

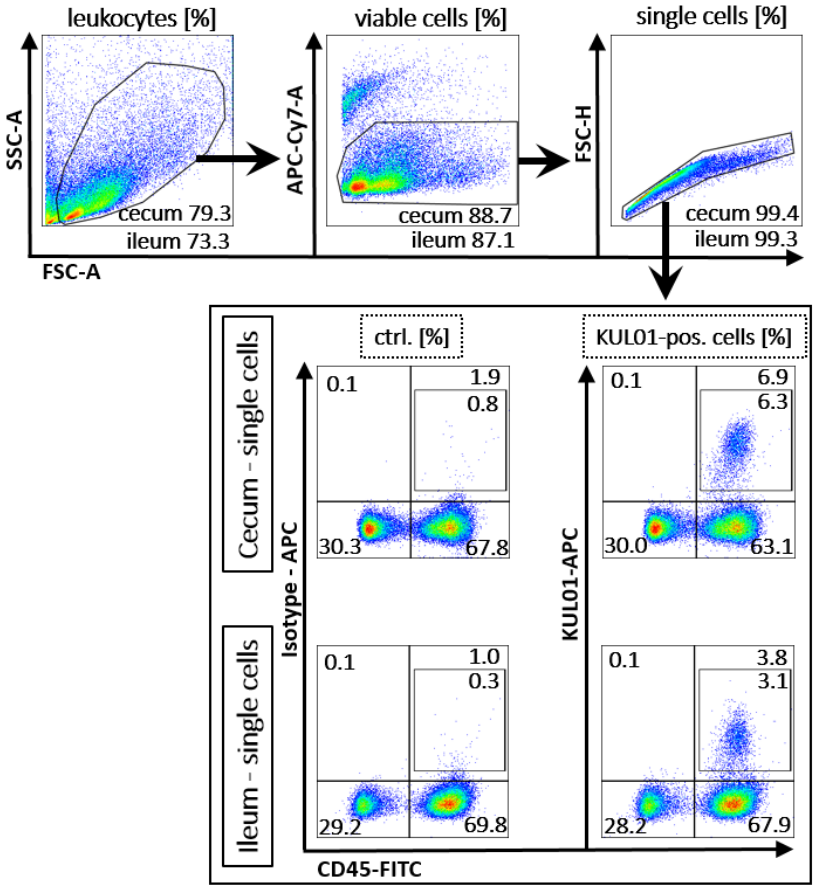

Figure 8: FACS based identification of gut derived macrophages (ileum and cecum); Data represent means [%] of 4 independent experiments

Cells were stained with the monoclonal antibody KUL01 (detects MRC1L-B) and CD45 (marker for leukocytes) and subsequently incubated with secondary fluorochrom-conjugated antibodies (FITC/APC). Absolute cell count of CD45+/KUL01+ -single cells in the cecum (mean/standard deviation): $1x10^7$/0.25. Absolute cell count of CD45+/KUL01+ -single cells in the ileum (mean/standard deviation): $0.43x10^7$/0.17.

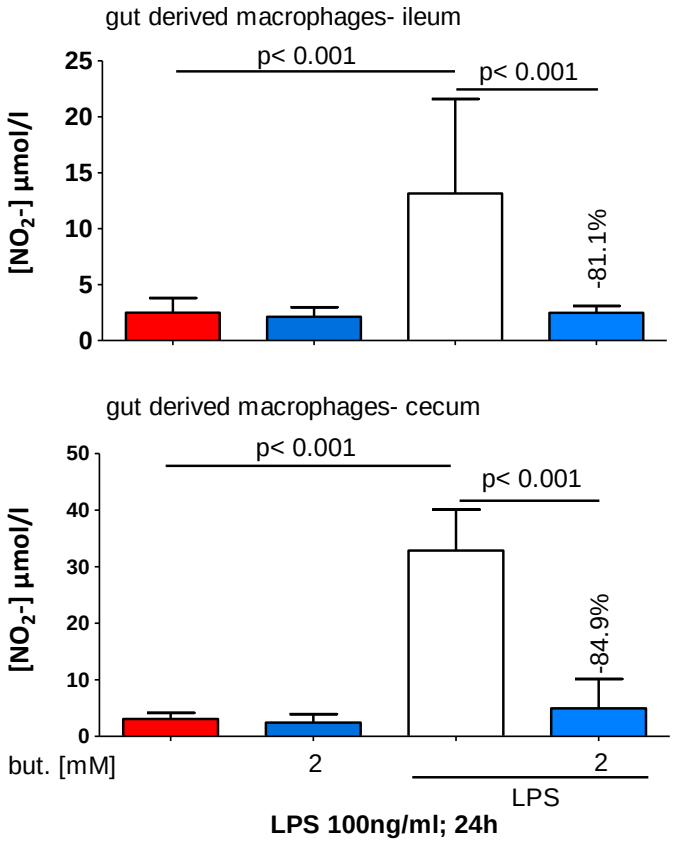

Figure 9: Response of gut derived macrophages (ileum and cecum) to LPS; n=4; 3 replicates

Cells were treated as described in Figure 2. A). Gut derived macrophages respond to LPS with the production of NO. Butyrate treated cells displayed reduced nitric oxide levels after stimulation with LPS in comparison. Data represent means and standard deviations. Percent decrease values were calculated against LPS treated cells. Statistics were performed using Wald Chi square.

5.2.3 Re-stimulation of primary cells

After the first stimulation (described under 5.2.1. and 5.2.2) primary cells were further cultured for 7 to 10 days in the absence of SCFAs and LPS, followed by LPS stimulation for 24h. Prior to stimulation the supernatant was tested for NO production (described under 5.2.3.1.1). Controls were included in this experiment.

5.2.3.1 Intermediate control

To exclude spontaneous production of NO in these aged cell cultures cell culture supernatants were analyzed for NO prior to LPS stimulation. The results were compared with untreated controls from the initial cultures (described under 5.2.1 and 5.2.2), here referred to as basic control.

The results show that NO concentrations were as low as the baseline controls. Deviations occurred particularly in monocyte derived cells treated with acetate (figure 9).

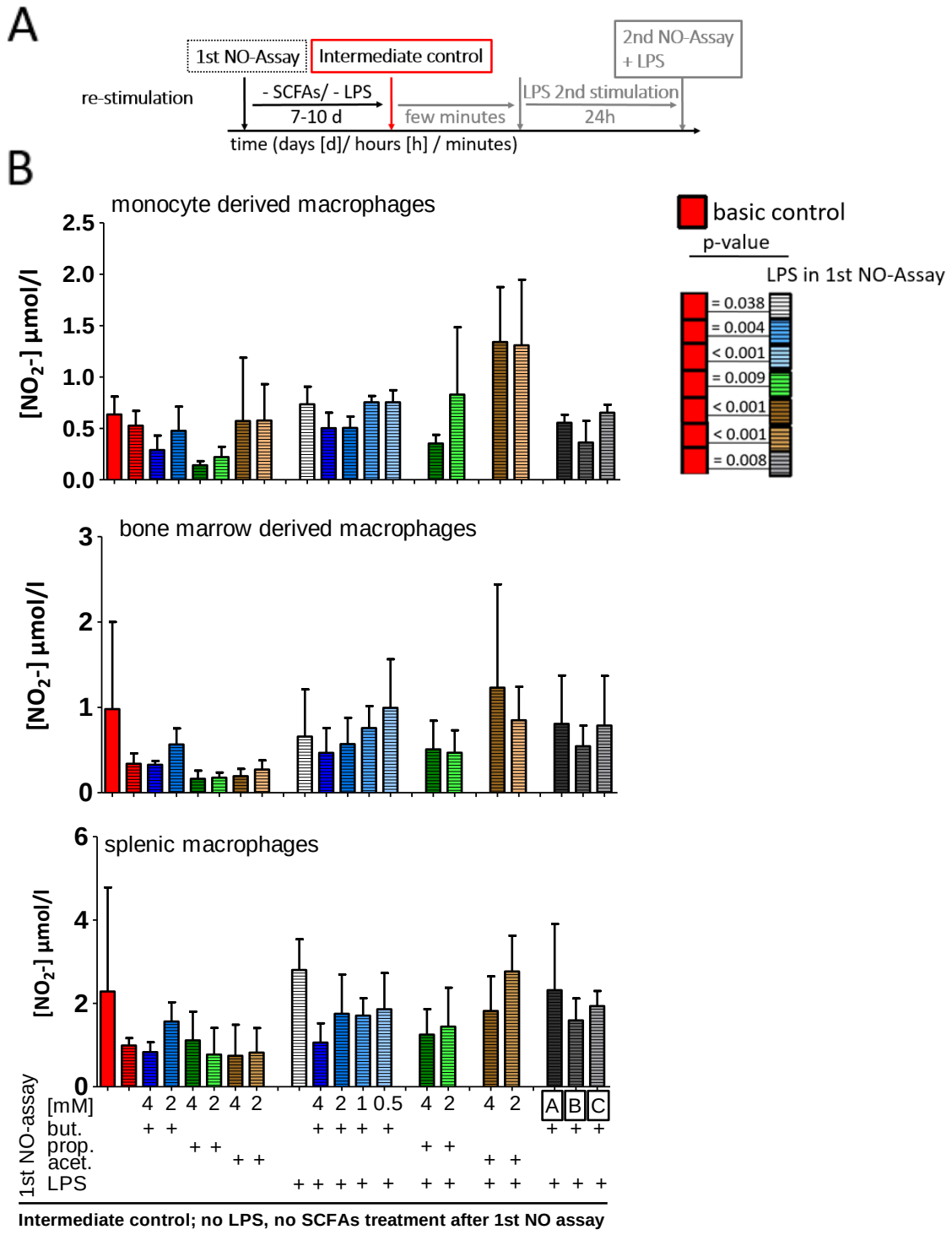

Figure 10: Intermediate control of primary cells derived from monocytes, bone marrow and spleen; n=3 in monocyte derived cells and macrophages derived from spleen; n=4 in bone marrow derived cells; 3 replicates

A) Treatment scheme B) Intermediate control: NO production after 7 to 10 days in the absence of butyrate and LPS. The supernantants of the cells from the first experiment (Figures 6 and 7) were tested for NO production in the absence of SCFAs and LPS. Comparisons were taken against the NO level of the untreated control (here referred to as basic control) in the first experiment. Data represent means and standard deviations. Statistics were performed using Wald Chi square. Only statistical relevant p- values are presented.

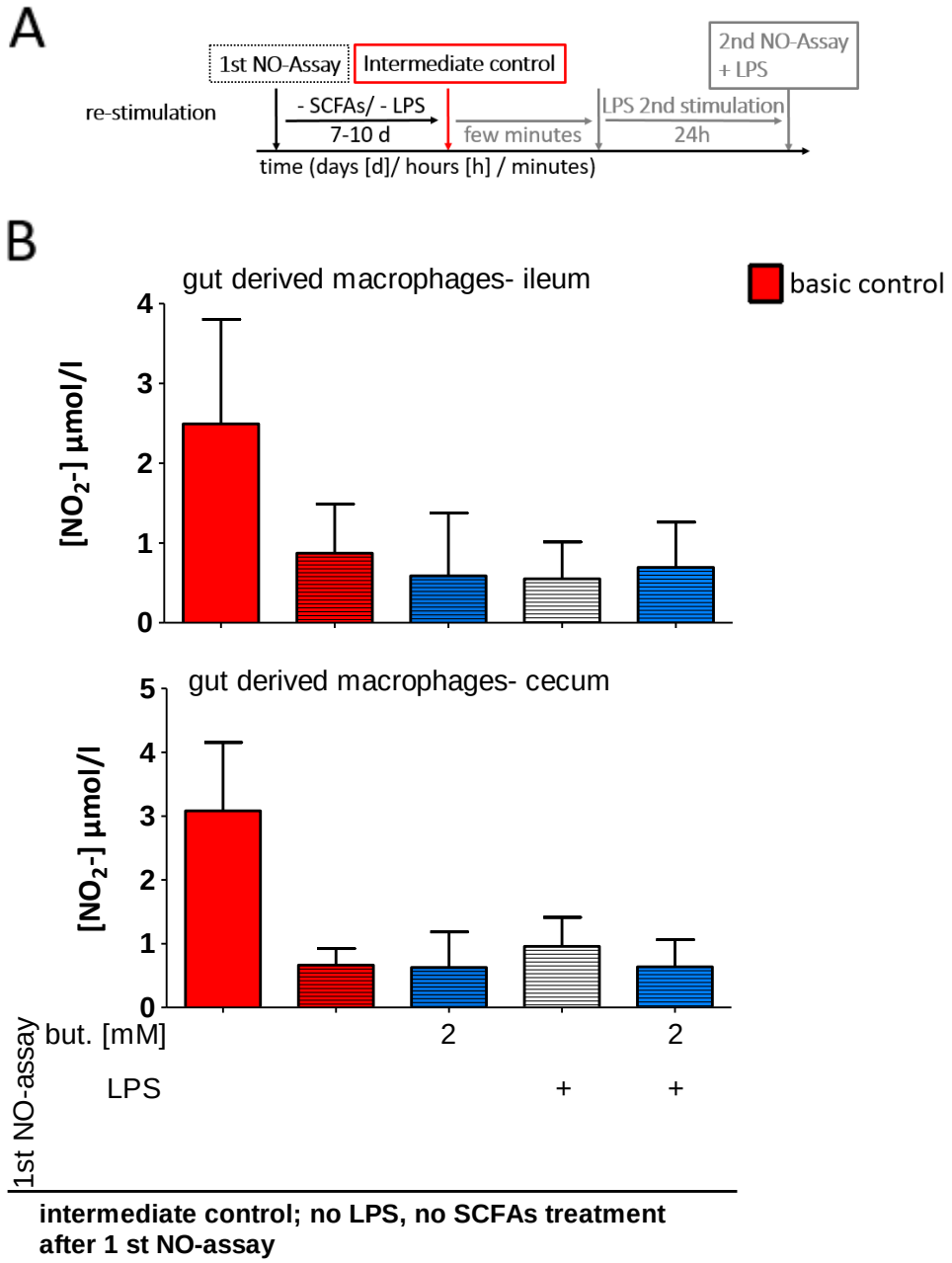

Figure 11: Intermediate control of gut derived macrophages (ileum and cecum) to LPS; n=4; 3 replicates

A) Treatment scheme B) Intermediate control: NO production after 7 to 10 days in the absence of butyrate and LPS. The supernantants of the cells from the first experiment (Figure 8) were tested for NO production in the absence of SCFAs and LPS. Comparisons were taken against the NO level of the untreated control (here referred to as basic control) in the first experiment. Data represent means and standard deviations. Statistics revealed no significant NO levels (data not shown). Statistics were performed using Wald Chi square.

5.2.3.2 Restimulation

Immediately after removal of the intermediate control, all cells including untreated and solely treated with butyrate (described under 5.2.1 and 5.2.2) were stimulated with LPS.

The NO values of the cells stimulated this way were compared with the basic control (described under 5.2.3.1 and the cells treated with LPS alone (described under 5.2.1 and 5.2.2), here referred to as LPS basic control.

Without exception, the NO values of the cells stimulated in this way exceeded the level of the basic control.

In cultures of cells derived from the cells from the bone marrow, the NO values were almost uniformly below the LPS basic control.

With few exceptions, macrophages derived from spleen responded with nearly the same amount of NO as the basic LPS control.

In monocyte derived macrophages, propionate-treated cells displayed the lowest NO levels in comparison. Macrophages derived from spleen treated with 4mM butyrate showed the lowest NO values compared to the LPS basic control.

Spearman rho calculation revealed a concentration dependence in the former butyrate treated cells derived from monocytes compared to the first part of the experiment described under 5.2.1. Compared to the first experiment the NO levels were in a reverse order.

In splenic cells, spearman rho values also showed a concentration dependence of butyrate effects in the spleen. This corresponded to the concentration dependence in the first part of the experiment (described under 5.2.1).

In some cases, NO production in splenic cells was higher than the baseline LPS control. This affected both cells treated with SCFAs and untreated cells (figure 12).

Intestinal cells that were treated exclusively with LPS in the first part of the experiment showed higher NO levels than the LPS basic control. Cells treated with butyrate and LPS showed lower values in comparison (figure 13).

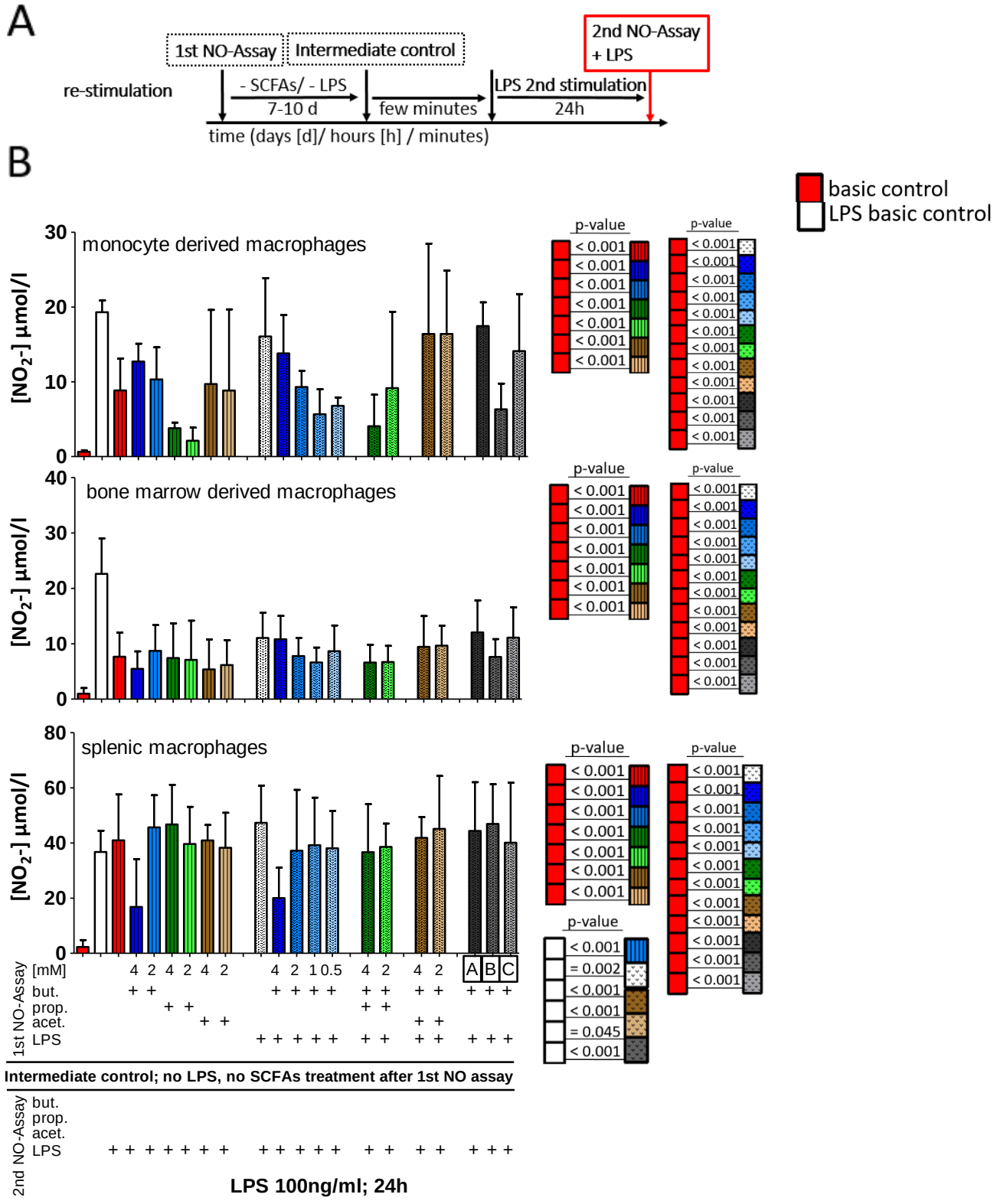

Figure 12: Re-stimulaiton of primary cells derived from monocytes, bone marrow and spleen; n=3 in monocyte derived cells and macrophages derived from spleen; n=4 in bone marrow derived cells; 3 replicates

A) Treatment scheme B) 2nd NO-assay: Cells were re-stimulated with LPS. Unstimulated controls from the first NO-assay were included. Comparisons were taken against the NO level of the untreated control (here referred to as basic control) and the LPS treated cells (here referred to as LPS basic control) in the first experiment. Data represent means and standard deviations. Statistics were performed using Wald Chi square.

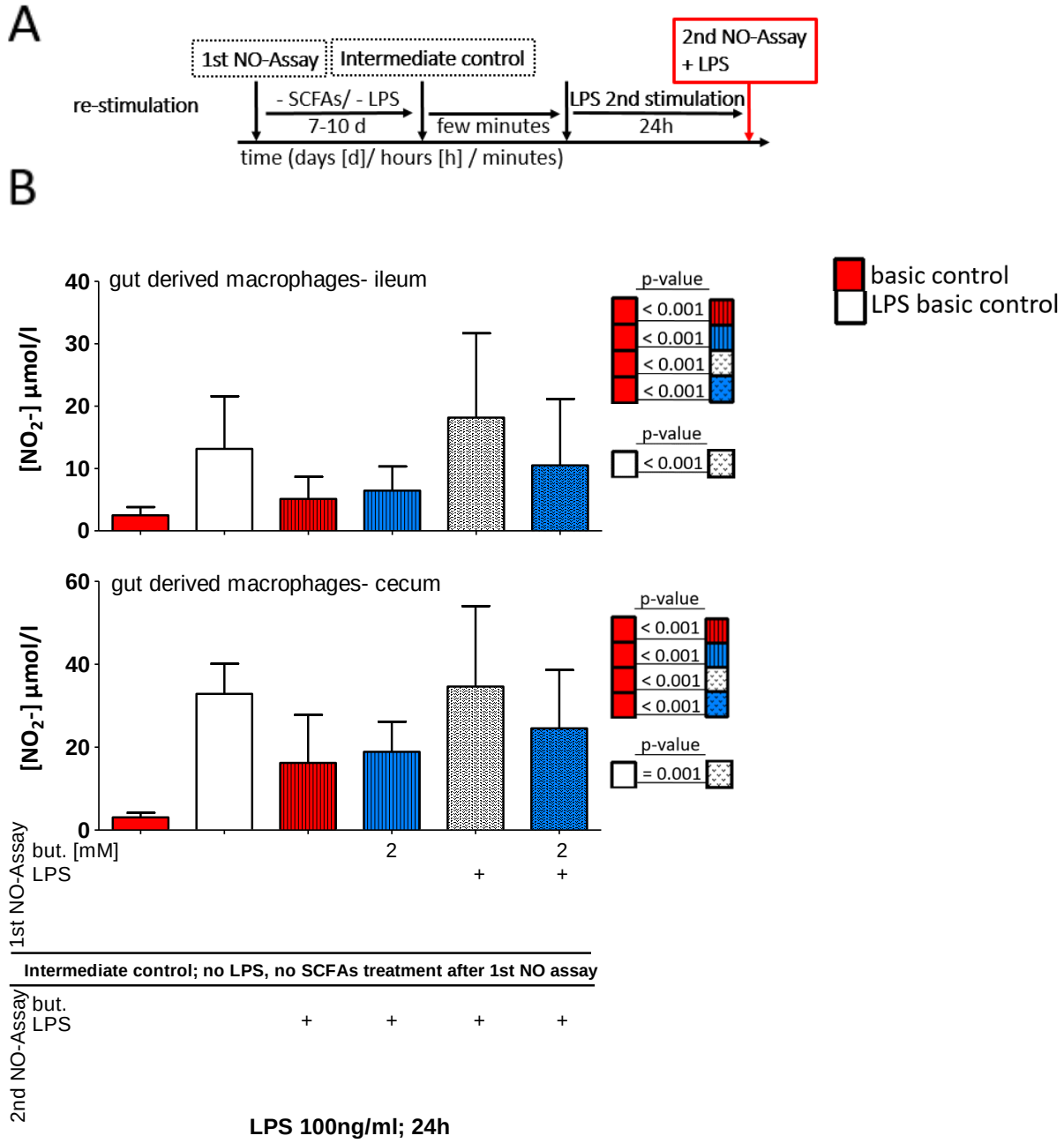

Figure 13: Re-stimulation of gut derived macrophages (ileum and cecum); n=4; 3 replicates

A) Treatment scheme B) 2nd NO-assay: Cells were re-stimulated with LPS. Unstimulated controls from the first NO-assay were included. Comparisons were taken against the NO level of the untreated control (here referred to as basic control) and the LPS treated cells (here referred to as LPS basic control) in the first experiment. Data represent means and standard deviations. Statistics were performed using Wald Chi square.

5.3 Cytokine expression

To test the effect of butyrate on cytokine expression levels, macrophages derived from spleen were isolated, cultured and stimulated as described under 4.5.1. RNA was prepared after 8 and 24h of LPS stimulation.

LPS induced the expression of IL-1ß, IL-6, IL-8, TNF-α and iNOS at 8h and 24h. Without exception, expression levels were lower in butyrate treated cells subsequently stimulated with LPS. Percent decrease values were calculated between controls and the respective LPS treated cells.

At 8h, the percent decrease values showed that the induction of gene expression by LPS is approximately the same in both groups.

The respective controls between the two time points were almost exclusively at the same level. In contrast, the 24h expression values were below the 8-hour values of the respective LPS-treated cells.

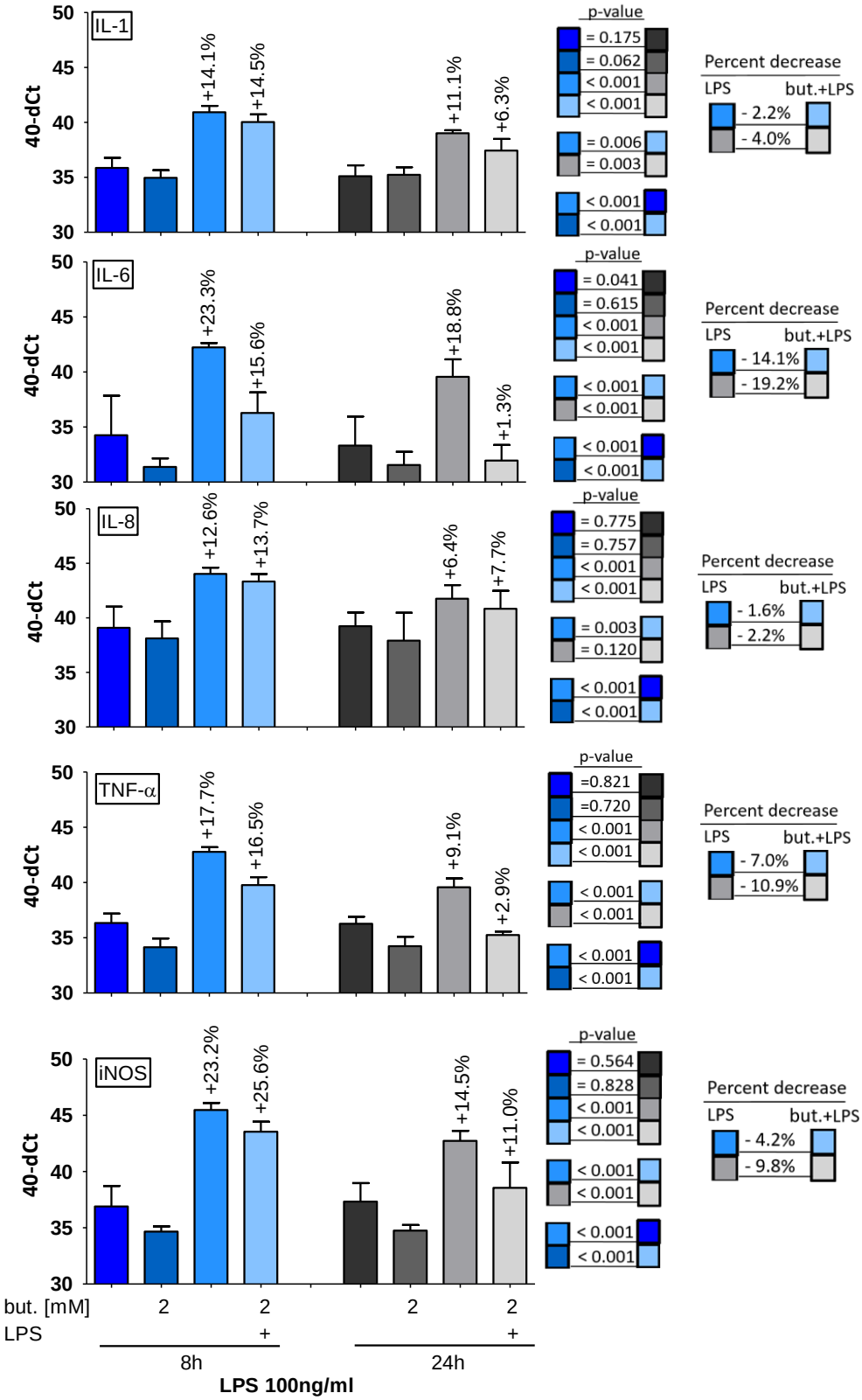

Figure 14: Cytokine expression of LPS stimulated macrophages derived from spleen; n=4;

Macrophages derived from spleen were pre-incubated with or without butyrate for 24h and subsequently stimulated with LPS in the presence or absence of butyrate. RNA was extracted after 8h and 24h of LPS stimulation. Quantitative Real-time PCR was performed to quantify the respective RNA expression levels. Butyrate reduced the gene expression of IL-1ß, IL-6, IL-8, TNF-α and iNOS. Data represent means and standard deviations. Percent decrease values were calculated against the respective control. Statistics were performed using Wald Chi square.

6 DISCUSSION

“All disease begins in the gut” – this sentence seems to contain a lot of truth based on previous research. Both chickens and humans have evolved in the context of microbial colonization and several host functions are mutually dependent on interactions with the microbiota (82). The influence of the intestinal microbiota has far-reaching systemic consequences in humans as well as in animals, which we are only beginning to understand. Modulating the gut microbiota through various pathways at all stages of chicken development has been proposed as a potential strategy to improve overall health and productivity and prevent adverse effects on gut health and the immune system (60, 83, 129, 377).

SCFAs are fermentation products that are mainly produced microbially in the intestine. In poultry farming, they are often used as a feed additive due to their performance-enhancing effect. Butyrate in particular has received more and more attention in recent years due to its immune-modulating effect, which also appears to be cross-species.

Macrophages display a heterogenous group of phagocytizing cells. They represent an essential part of the innate immune system and have sentinel functions. On the one hand they protect the body from harmful pathogens promoting inflammation, on the other hand they play an important role in maintaining tissue homeostasis, which is closely linked to anti-inflammatory abilities.

This study aimed to provide insights into the effects of short-chain fatty acids, particularly butyrate, on avian macrophages. Functional tests under the influence of SCFAs in form of NO-assays were carried out at first with the chicken macrophage cell line HD11 and the murine macrophage cell line RAW 264.7 followed by assays on primary chicken macrophages derived from different tissue sources (blood, spleen, bone marrow, ileum, cecum). In order to take a closer look at these influences, the cytokine expression of resident splenic macrophages was examined.

6.1 NO-Assay-cell lines

Macrophages produce NO as a part of defensive response to an LPS stimulus (348).

The NO produced breaks down into nitrite and nitrate in a ratio of 3:2. A proven readout system, the Griess reaction, was used to measure nitrite production after LPS stimulation (348). The resulting diazo dye leads to a change in the optical density. By comparing the measured values

with a standard nitrite regression line, quantitative conclusions were drawn in the subsequent experiments as described by van den Biggelaar et al. (348).

6.1.1 HD11 cell line

Cell lines are widely used to conduct translational studies and offer experimental advantages that do not require animal testing.

In a first set of experiments, the well established chicken macrophage cell line HD11 (378) was used as a model system to further investigate the influence of SCFAs on chicken macrophages.

First, to determine an appropriate LPS concentration for subsequent experiments, LPS was tested in a log2 scale on HD11 cells, starting with a concentration level which can also be found in chicken HD11 literature (121, 124). Cells treated with LPS for 24h responded by producing NO in a dose-dependent manner, which were also shown repeatedly in subsequent experiments.

Next, to determine a suitable time point for the following experiments, the cells were treated with LPS and the NO produced was measured at different time points. Two different LPS concentrations were used and the cells increased NO production continuously over a 24h period. A dose and time-response relationship was most evident after 12h-24h at a LPS concentration of 5ng/ml in this experiment. After 48h, clear morphological changes such as cell shrinkage and detachment could be observed. As previous studies showed, this could be due to cell death, possibly caused by the high amount of NO in the supernatant (B. Kaspers, personal communication, April 23, 2021).

Due to better feasibility and comparability with the existing literature in chickens for HD11 (86, 120) and HTC (90, 121) macrophage cell lines in experimental settings with SCFAs, the following experiments were read out after a 24h stimulation period with LPS. Thus, an assay system was established which allowed detailed studies of anti-inflammatory properties of SCFAs.

To investigate the effects of SCFAs on HD11 cells, the experiment was modified in the next step. Prior to stimulation with LPS, cells were incubated with SCFAs for 24h. Different LPS concentrations were used as well as different concentrations of SCFAs and the amount of NO produced was measured after 24h. A SCFA-concentration of 2mM was used as reported in chicken literature for HD11 cells (86, 120, 121, 379) and the HTC cell line (121, 122).

Butyrate and, to a lesser extent, propionate inhibited nitric oxide production of LPS-stimulated HD11 cells. Acetate showed only a marginal inhibitory effect in comparison. The inhibitory

effect was most evident at higher concentrations of LPS for all SCFAs. These findings are consistent with Zhou, who examined the effect of SCFAs on NO production in the chicken macrophage cell line HTC and demonstrated the potent inhibitory effect of SCFAs (90).

6.1.2 RAW 264.7 cell line

To compare the effect of SCFAs on chicken cells with published observations in mammalian systems the murine cell line RAW 264.7 was treated in the same way as HD11 cells.

Since butyrate in particular has already shown suppressive effects on NO production in the mammalian literature (380-382), RAW 264.7 cells, were treated with butyrate followed by additional stimulation with LPS. Compared to cells stimulated with LPS alone, butyrate-treated cells also displayed a reduced NO production, confirming the findings of Chakravortty et al. and Park et al. (380, 382).

In all cell line experiments performed in this study, butyrate alone did not exert any NO-inducing effect on the cells. This is in contrast to Dias et al., who examined the effects of butyrate (0.01, 0.1mM) on NO production of endothelial cells (383) and Morikawa who found enhanced NO-Production in the endothelial cell line END-D due to butyrate treatment (37). Moreover, butyrate (0, 20, 40mM) enhanced NO-production in the murine macrophage cell line J774.16 stimulated with different yeast strains in a concentration dependent manner (384). Cell line-specific intrinsic differences in the regulation of NO inducing pathways could be responsible for this discrepancy, as these can be found in human cancer cell lines even when they are assigned to the same organ types (385). In chickens, macrophage cell lines of different organic origins (HD11; MQ-NCSU) responded to the same stimuli (LPS, IFN-y) with different and contrasting levels of NO production (386). Dose-dependent effects of butyrate could also be the cause (387). Moreover, observational studies report reversible changes in cell shape, describing a more elongated type in butyrate and to a lesser extend in propionate treated cell lines, especially at higher SCFAs concentrations (388-390). Comparable results were made in this study.

Furthermore, due to the physicochemical properties as well as the absorption and transport mechanisms, the same SCFA can have multiple effects on the same cells (116).

Not least because of this, further studies including deep sequencing methods are necessary to decipher the exact effects of SCFAs in each experimental approach with cell lines.

6.2 NO-Assay-primary cells

Since studies with immortalized cell lines only reflect the situation in non- transformed cells to a limited extent, primary cells were isolated and cultured.

Several studies discussed in the human and murine literature indicate local and systemic immunosuppressive functions of SCFAs on various types of primary immune cells, including macrophages (116). SCFAs are mainly produced in the gut by commensal bacteria. In particular butyrate was shown to exert anti-inflammatory effects. The spectrum of macrophage functions comprises pro-inflammatory properties as well as anti-inflammatory effects. The functions of macrophages are highly diverse and dependent on a range of tissue specific factors. Therefore, regulation of macrophage function may represent a key element in regulating immune answers.

To gain more insight into the interaction of gut derived SCFAs and macrophages, blood, bone marrow and spleen were used as sources for chicken macrophages and examined in the context of SCFAs treatment. Especially butyrate was investigated, besides propionate and acetate. Subsequently, the effects of butyrate were further examined on intestinal (ileum, cecum) derived macrophages, since SCFAs production appears mainly in the chicken intestine. Animals aged 8–9 weeks were used for all experiments with primary cells. At this point the development of the immune system in the intestine is largely completed (55).

6.2.1 Stimulation of monocyte derived macrophages / splenic macrophages / bone marrow derived macrophages

As parts of the innate first line of immune defense, macrophages are known to respond quickly after stimulation in mammals (391) and avian species (322). Moreover, immunological reactions often follow strict time courses (392).

To determine suitable time points for subsequent experiments, a time course experiment was performed as described for HD11 cells. Primary cells derived from blood, spleen and bone marrow were treated with or without butyrate followed by LPS stimulation for various times. Butyrate was used at a concentration of 2mM as used in the chicken literature (see references above). To stimulate the TLR4 system, LPS was applied at a concentration of 100ng/ml as already described in chicken primary macrophage literature (372, 393).

Comparable to HD11 cells, untreated cells and cells incubated exclusively with butyrate did not produce NO at any of the evaluated time points, regardless of the tissue source. This indicates that butyrate alone does not induce NO production and that NO production is solely

due to the LPS stimulus. This is of biological relevance since TLRs have been found to recognize nutrients and microbial metabolites in addition to PAMPs, which can also lead to a special kind of inflammation, called meta-inflammation due to nutrient or metabolic excess (60, 82, 394-396). As discussed earlier, butyrate enhanced the NO production in different cell line types (see references above). However, these cells may therefore use different cell signaling pathways.

In this study, a 4h treatment with LPS or butyrate plus LPS displayed significant NO production compared to the untreated controls in most cases. P-values in monocyte derived macrophages were close to significant ($p= 0.056$) in butyrate treated cells. After 6h this effect was evident in all primary cells. NO production increased significantly under the influence of LPS over a period of 48h, in the absence or presence of butyrate (spearman rho $p< 0.001$ in all tissues). After an incubation period of 2h, NO levels were below detection limit in prior experiments although cellular changes were already observable under the microscope (data not shown).

This is largely consistent with time course studies in mammalian primary macrophages stimulated with LPS (397, 398). Butyrate supplementation resulted in a reduction of NO production at every time point measured compared to the respective LPS-control, as revealed by percent decrease values. Nitric oxide is involved in many physiological and pathophysiological processes displaying adverse functions (399-401). In contrast to high concentrations of NO, lower levels of nitric oxide were found to exert anti-inflammatory effects (402, 403) probably through NO gyanylyl cyclase/cyclic guanosine monophosphate (cGMP) signaling (403, 404). Niedbala et al. found that NO is involved in Treg (NO-Tregs) differentiation in mammals in a cGMP independent way (399). Therefore, the use of butyrate could be relevant in controlling inflammation in chickens. The time course showed that macrophages did not completely stop NO production due to butyrate treatment but reduced iNOS activity.

Detached cells were found after 48h of LPS stimulation. This might be due to a decreased cell viability as discussed earlier for the HD11 experiments. Thus, the 24h time point was selected in the following experiments.

Information on the effects of acetate and propionate on NO production by primary chicken macrophages is scarce, although these SCFAs reach the systemic circulation in this species probably in higher concentrations than butyrate (30, 184). Using a spectroscopy-based method (proton nuclear magnetic resonance) Saint-Martin detected acetate in the lungs of GF housed chickens, suggesting an endogenous production (30, 184).

Dose dependent contrary effects of SCFAs are reported by several authors (405-408).To evaluate potential concentration dependent contrary effects in chicken primary macrophages, cells were treated with different concentrations of SCFAs in the following experiment.

Primary cells were pre-treated with propionate and acetate in two different concentrations (2mM and 4mM). In addition, butyrate was applied in a log2 scale. After 24h of LPS treatment, all three SCFAs exerted a significant inhibitory effect on NO production by primary macrophages. Similar to the results in SCFAs treated HD11 cells, percent decrease revealed that butyrate and to a lesser extend propionate significantly inhibited NO production of LPS stimulated primary cells regardless of the tissue origin. Acetate treated cells displayed a minor inhibitory effect in comparison. Concentration-dependent effects of butyrate were found on bone marrow derived and splenic cells (spearman rho $p< 0.001$). This effect was not significant in monocyte derived macrophages. However, percent decrease calculation revealed a slight concentration-dependent difference in NO production in monocyte derived macrophages.

Higher concentrations of propionate and acetate caused a less pronounced effect than the corresponding lower concentration in monocyte derived cells as well as in bone marrow derived cells.

Propionate treated cells displayed a concentration dependent inhibitory effect on monocyte derived and splenic macrophages. This effect was slightly reverse in bone marrow derived cells and might be due to technical aspects such as turbidities interfering with the optical density measurement.

Acetate treated cells displayed a less concentration dependent effect in comparison.

Findings reported here are partly consistent with Chang et al. (409) and Fernando et al. (410) who examined the NO production in mammalian bone marrow derived cells. Concentration levels of 1mM displayed no decrease in NO production in propionate as well as in acetate treated cells (409). Significant differences were evident at 2mM (410).

Investigating the effects of acetate, propionate and butyrate on the mammalian cell line RAW 264.7 and bone marrow derived macrophages Park et al. also found concentration dependent effects for propionate and butyrate treated cells in contrast to acetate treatment (382). This could be due to different mechanisms of absorption of acetate based on its physicochemical properties. Moreover, the mechanisms of actions of SCFAs on chicken primary macrophages might differ (116, 162).

SCFAs exert their functions through several mechanisms. Thus, different types of SCFAs can have different effects on the same cell type (116). The repertoire of SCFAs mechanisms of action includes the interference with NF-kB activation in the order of butyrate > propionate > acetate (116). The same ranking can be found regarding histone deacetylase (HDAC) inhibition (116). Histone deacetylases regulate chromatin structure and therefore gene expression through removal of acetyl groups of histones. Butyrate treatment has been shown to increase the acetylation of the NF-kB gene through its function as HDAC inhibitor resulting

in decreased NF-kB levels (411). NF-kB is involved in the upregulation of pro-inflammatory molecules such as iNOS (412, 413).

Singh et al. (414) and Arpaia et al. (415) found that acetate has no HDAC inhibitory function, unlike butyrate and propionate. In mammals, butyrate has been shown to decrease NO levels by acting through HDAC inhibition (116). A reverse ranking was observed for the affinity for G protein-coupled receptors. Acetate has been shown to exert pro-inflammatory effects through GPR41/GPR43 activity (116). On the other hand, butyrate displayed anti-inflammatory effects including the inhibition of NO production through the activation of GPR43 (416). In the chicken, more than 20 paralogs of GPR43 have been found by genome analysis but their mechanisms of action are still unresolved (30).

Contrasting and paradox effects of SCFAs are described in the literature and discussed as being due to the individual metabolism of the target cells (202, 417-419).

For example proliferating as well as differentiating cells display a different cellular metabolism (420) and regulating immunometabolism is an upcoming field of research (421-424). Even in cell culture systems derived from one organ macrophages display a heterogenous group with different phagocytic activity (235). Predominantly glycolytic pathways are involved in phagocytosis in macrophages (425). Moreover, contrary effects were found in monocytes and macrophages due to SCFAs treatment (426).

Nitric oxide-inducing effects of acetate have been observed in mammalian endothelial cells (427) as well as in alveolar macrophages (428). These effects might also be due to differential regulation of TLR4 responses to LPS stimuli (116). Concentration dependent adverse effects have been reported in cells treated with SCFAs (116, 429). Moreover, due to their pysicochemical properties, SCFAs can accumulate in cells (202, 419). Dose dependent effects might therefore also follow a time course.

These effects could be of biological relevance. The amounts of butyrate found so far in the chicken body outside the intestine are lower than those of propionate and acetate (184) Nevertheless, butyrate seems to have a stronger inhibitory effect at the same molarity.

Splenic cells seem to differ from monocyte and bone marrow derived macrophages. This could be due to differences in cell status since monocytes differentiate upon extravasation into macrophages, achieving functional maturation in a tissue dependent way (430). So far chicken tissue resident macrophages are believed to be replenished from bone marrow derived precursors (monocytes) entering the blood circulation (229).

Reversible changes in cell shape could be observed in primary cells treated with butyrate and propionate similar to those in HD11 cells (data not shown). Using a micropatterning approach, McWorther demonstrated that elongation of cell shape itself, similar to the microscopical

findings here (data not shown) leads to the expression of M2 phenotype markers and reduces the secretion of inflammatory cytokines (431). Cabanel et al. found that the use of Trichostatin A, a known HDAC inhibitor led to a shape transition from the macrophage pancake-like shape into an elongated morphology. He et al. also found a correlation of this atypicaly elongated phenotype to a mixed M1/M2 phenotype based on the cytokine expression (432). Regulation of macrophage by polarization towards the anti-inflammatory M2 type by butyrate is already described in mammals (397, 433, 434). In contrast, Huang et al. found that butyrate and propionate but not acetate inhibit the M2 polarization in murine alveolar macrophages (73). Moreover, Foey reports differential regulatory functions in cytokine production of butyrate on M1 and M2 macrophages (435). In the chicken, first steps are made towards M1/M2 characterization (436) but the existence of M1/M2 phenotypes in chickens is not yet clear.

A color difference in the cell culture supernatant due to changes of the pH value provided evidence of an altered cell metabolism in butyrate and propionate treated cells. This was absent in acetate treated cells (data not shown). In murine macrophages, Schulthess et al. observed a decreased extracellular acidification rate due to butyrate treatment and found that this was referred to an inhibition of mTOR (437). mTOR is a master regulatory protein of several physiological processes including cellular metabolism (438). In the present study, butyrate treated macrophages also displayed an altered extracellular acidification rate. Moreover, in contrast to M2 macrophages, M1 polarized cells display an enhanced NO release due to an altered L-arginine metabolism in mammals (211).

However, percent decrease revealed that butyrate had the strongest inhibitory effect on monocyte, spleen and bone marrow derived macrophages. Therefore, only the effect of butyrate was further investigated.

In *vivo*, macrophages are often confronted with conflicting stimuli (439) and their M1/M2 polarization can switch vice versa in mammals (211). In order to simulate different situations, different approaches have been developed in *vitro*.

Treatment with butyrate inhibited NO-production in every approach regardless of the experimental setup. Butyrate and LPS treated primary cells displayed a significantly lower NO production compared to the LPS-control in all culture systems. The percent reduction differ depending on the organ of cell origin. This could be due to differences in cell status as discussed earlier or technical aspects.

The use of butyrate has proven to be therapeutically effective in several studies with inflammatory diseases (440-444). To simulate an ongoing inflammatory process cells were incubated with LPS for 4h. Subsequently, butyrate was added and NO levels were evaluated 20h later.

LPS stimulation for 4h leads to a significant release of NO in most cases (figure 5). Adding butyrate to the cells 4h after LPS treatment seemed to downregulate the NO production in these proinflammatory driven macrophages. NO values were significantly lower compared to the LPS control. A similar experimental approach showed reversed changes in cellular respiration in a lymphoblastoid cell line compared to the control treated with LPS alone (445). Chakravortty et al. used a similar approach in RAW 264.7 cells and found a decreasing effect on NO Production due to butyrate treatment in cells pre-exposed to LPS for 6h (380). This indicates that pro-inflammatory driven macrophage responses can still be modulated by butyrate after initial PAMP mediated cell activation. Therefore, butyrate could also be of therapeutical use in ongoing inflammations in the chicken. Moreover, this supports the assumption that chicken macrophages may also display a functional versatility similar to that found in mammals.

Preconditioning with butyrate for 24 hours followed by LPS stimulation in the absence of butyrate also displayed a reduced NO production. This indicates that butyrate might have a longer lasting suppressive effect. This is partly in contrast to Chakravortty et al., who pre treated RAW 264.7 cells with butyrate for various times (380). Pretreatment with butyrate did not affect the LPS induced NO production in this case. This could be due to the comparatively short pre-incubation period of 6h or differential expression of TLR4 in this cell line. Schulthess et al. repored that in contrast to a long-term pre-exposure, a short-term exposure (3h) to butyrate of mammalian macrophages enhanced phagocytic activity (437). Therefore, further studies including long-term approaches are required.

6.2.2 Stimulation of intestinal macrophages (ileum / cecum)

The antigen mix of nutrient components, microbes (commensal and pathogenic) and microbial metabolites as well as the hosts own structures represents a challenge for the local immune system. Live bacteria migrate across local defense barriers in both humans (125) and chickens (127, 128). The microbiota trains and stimulates the immune system locally and systemically at all stages of life in the chicken. Local innate and adaptive immune responses have to be balanced to keep homeostasis. Moreover, the major route of pathogen entry in the chicken is the GI-tract and severe inflammations may occur locally (178).

The largest proportion of macrophages in humans can be found in the lamina propria of the intestine (91). There they display a unique, mostly anergic phenotype, despite keeping their phagocytic and bactericidal properties (91, 232). In mammals SCFAs are mainly produced in the intestine, where they contribute to the tolerogenic milieu (282). In the chicken, acetate seems to contribute to the tolerogenic milieu by regulating Tregs in the intestine (205).

Therefore, macrophages from intestinal sections, especially the cecum are most likely to be influenced by SCFAs. Chicken intestinal macrophages are functional at hatch. Boodhoo et al. isolated primary macrophages from 3-week-old chickens. Cells derived from duodenum, jejunum, ileum and cecal tonsil produced NO in response to Clostridium perfringens treatment (307). Since maturation of the intestinal immune system in the chicken takes place until 8 weeks of age (55) 8 week old animals were used in this study. A modified isolation protocol was used to prepare macrophages from the ileum and cecum. Initially, intraepithelial lymphocytes were prepared following an established protocol (446). The IELs were either used for additional questions or discarded. Subsequently, the lamina propria was enzymatically digested and macrophages were identified using the KUL01 antibody (detecting MRC1L-B) in combination with CD45.

Like primary cells from blood, spleen and bone marrow, cells were pre-treated with butyrate followed by additional LPS stimulation. Treatment with butyrate resulted in significant reduction of the NO-production compared to the LPS-control in ileum as well as in cecum derived cells. Intestinal macrophages responded with NO production to LPS stimulation and therefore did not display an anergic status. This contrasts with the mammalian literature, where intestinal macrophages are classified as inflammation anergic (91, 232). These findings may be important regarding butyrate as a potential therapeutical tool to control excessive inflammation in the chicken gut.

The response of macrophages largely depends on the prevailing environment (213). Therefore, withdrawal of the tolerogenic in *vivo* environment during the initial in *vitro* culture may have been responsible for this NO production since intestinal cells were first incubated for 72h in order to remove accompanying cells. However, chicken intestinal macrophages stimulated immediately after preparation, also responded with NO production (307).

6.2.3 Re-stimulation of primary cells

The reduced NO production and metabolic/morphological alterations could also be due to cytotoxic effects or death induced by SCFAs.

To investigate potential cytotoxic effects of SCFAs, all cell culture systems were further cultured for 7-10 days in the absence of SCFAs and LPS followed by re- stimulation with LPS only. SCFA and untreated controls were included in this follow-up experiment. To find out whether NO production was solely due to re-exposure to LPS, the supernatant of each batch was sampled prior to re-stimulation to quantify background NO levels (here referred to as intermediate control). A similar approach was used by Verwoolde et al. who investigated chicken macrophage memory functions (251).

The NO levels of the untreated cells (no LPS/ no SCFA) and LPS treated cells from the first experiment served as reference. To be able to distinguish the NO levels of these cells in the first experiment from subsequent experiments those NO levels were renamed as basic control and LPS basic control respectively.

NO levels of the intermediate controls were generally at least at a similar level as the basic control indicating that no nitric oxide was produced in the absence of LPS.

The NO levels of the completely untreated cells from the first experiment were lower for each organ than the levels from the second experiment. This suggests that the completely untreated cells from the first and second experiments might have an altered, slowed down metabolism, This is probably due to culturing methods and is consistent with Verwoolde et al. who also included the untreated controls in his follow-up experiment (251).

In particular, samples previously treated with acetate and LPS displayed a higher NO value compared to the baseline control and the LPS basic control in some cases. Besides technical aspects, this might refer to an increased metabolism due to co-treatment with acetate, especially since the acetate controls were at least the same level with the initial control, if not lower. Acetate is endogenously produced in humans (447) as well as in chickens (30, 184). Considering long term effects of SCFAs treatment, this could be of biological relevance. Moreover, as mentioned earlier, acetate treatment resulted in elevated NO levels in murine alveolar macrophages (428).

After evaluation of NO levels of the intermediate controls the supernatant was completely discarded and cells were stimulated with LPS for 24h.

NO levels of stimulated cells were compared against the basic control and the LPS basic control.

Without exception, the macrophages from the first part of the experiment responded to the LPS stimulus by producing NO. This is consistent with Verwoolde et al. who examined trained immunity in chicken macrophages (251) and inconsistent with the avian literature examining tolerance mechanisms upon repeated stimuli, also referred to as endotoxin tolerance (308, 309, 448). Although exact mechanisms still remain to be further elucidated, so far tolerance and resistance to the same antigen in mammals depends on the magnitude, duration (316), age and time course of toxin application (449, 450).

In monocyte-derived cells, propionate appeared to have a long-lasting suppressive effect. However, cells treated with acetate and butyrate were easier to re-stimulate than the completely untreated control. Nevertheless, NO levels of butyrate and propionate treated cells were lower than the LPS positive control. This might also indicate that SCFAs interfere with the mechanisms of trained innate immunity leading to reduced responses upon re-stimulation.

The concentration dependence of cells treated with butyrate and LPS in the first course was mainly observed in monocyte-derived and splenic cells. In macrophages derived from spleen, this corresponded to the concentration dependence in the first part of the experiment. Here the cells reacted with less NO at a higher butyrate concentration.

In monocyte derived cells, the correlation was the opposite during the second stimulation. The dose-dependent side effects mentioned earlier could be the reason for this phenomenon. In addition, at a concentration of 4mM butyrate seemed to further inhibit the NO production in splenic cells. This also indicates longer lasting effects of SCFAs. These substances also pass through the cells via diffusion (116). Cell type-specific differences in membrane properties could lead to different diffusion rates and possible reabsorption, since membrane fluidity of macrophage membrane is dependent on environmental stimuli (451) and cholesterol metabolism (289). Butyrate treated RAW 264.7 cells displayed reduced membrane cholesterol (452).

Therefore, kinetic studies with organ specific cells would be required since metabolic pathways might differ dependent on the tissue (447) and the expression and distribution of SCFAs transporters needs to be further elucidated in the chicken.

However, compared to the other organ systems investigated, especially splenic macrophages displayed higher NO values upon re-stimulation in most cases. In splenic cell cultures, several NO levels exceeded the NO level of the LPS basic control. These findings are partly inconsistent with Verwoolde et al.. As discussed earlier, in this study unstimulated controls as well as solely with LPS stimulated cells did not respond with higher NO levels during the second stimulation experiment (251). Innate memory functions, such as upregulation of TLR4 could be responsible for this (453). However, cells that did not receive LPS in the first part of the experiment also showed NO production that partially exceeded the level of the LPS basic control. As already discussed, this could be due to the maturation status of the cells, as well as environmental factors in the spleen before isolation.

In bone marrow derived cells, NO levels upon second stimulation did not exceed the NO levels of the LPS basic control. This is consistent with Verwoolde et al. (251). At least in his study, the unstimulated control reached a similar NO level compared to the LPS treated chicken bone marrow derived cells. This is inconsistent with the mammalian literature (454) and could also be due to an altered cellular metabolism, although the exact mechanisms remain to be further elucidated. Mechanisms of tolerance such as downregulation of TLR could also be responsible for this phenomenon (455).

Interestingly, ileum and cecum derived cells treated with butyrate and LPS during the first stimulation showed similar NO levels upon second stimulation. Solely with LPS stimulated cells

displayed significantly higher NO values than after the previous experiment. This suggests that innate memory might also be present in gut derived macrophages. Gut derived macrophages are most likely to be influenced with repeated stimuli, since bacteria translocate in both mammals and chicken. Moreover, macrophages sample the intestinal lumen forming transepithelial dendrites (232). Regulation of intestinal homeostasis through butyrate might therefore be essential for chicken gut health.

In summary, all cells responded with the production of NO in the re-stimulation experiment with LPS. Thus, the inhibition of nitric oxide synthesis during the first stimulation is not due to cell death. However, differences in NO levels were also observed, which may implicate long term effects of SCFAs. Further studies are required to investigate the full spectrum of SCFA treatment.

6.2.4 Cytokine expression

As a prerequisite to a successful immune answer macrophages communicate with other immune cells through the production of cytokines. Those molecules serve to recruit and activate for example B- and T-cells as well as heterophils which often results in the typical signs of inflammation such as swelling and sometimes tissue destruction (456). Since birds lack lymph nodes, the spleen represents an important and accessible immunological organ in the chicken, which is also involved in responses to a range of infections (457, 458). Splenic macrophages are tissue resident cells and therefore represent a high degree of maturity. Moreover, the impact of SCFAs in the chicken spleen is understudied (30).

Contrasting information about pro-inflammatory gene expression due to butyrate treatment exists in the mammalian literature. Butyrate has been found to synergistically act with LPS and thereby enhance cytokine expression. For example, IL-8 expression was increased in epithelial cells following butyrate treatment in a dose dependent manner (459). IL-6 expression was increased following butyrate application in esophageal carcinoma cells (460). Furthermore, butyrate synergistically acted with LPS to enhance macrophage inflammatory protein-2 in rat small epithelial cells (461). On the other hand, numerous studies confirmed the anti-inflammatory effects of butyrate on cytokine expression levels (434, 437, 462-465). Moreover, since butyrate is known to exhibit anti-proliferational effects (419, 466-468) normalization of RNA amounts prior to qPCR testing is independent of cell counts.

To investigate the effect of butyrate on chicken macrophages derived from spleen the expression levels of the pro-inflammatory cytokines IL-1ß, IL-6, IL-8, and TNF-α and iNOS expression were investigated.

Cells were pre-treated with or without butyrate for 24h followed by additional LPS stimulation in the absence or presence of butyrate. Similar approaches are also used in the mammalian literature (469). In contrast to germ-free animals, butyrate can be found in lung tissue of conventionally housed chickens (30, 184) which suggests a permanent exposure of systemic macrophages to butyrate. Two time points post LPS exposure (8h and 24h) were chosen, since cytokine expression levels and inflammatory processes often follow time courses (119, 392, 470).

Without exception, LPS significantly induced the cytokine expression compared to the untreated control. In butyrate plus LPS treated cells the gene expression was significantly reduced in most cases. After 24h there was no significant difference of IL-8 expression between the LPS treated cells and the butyrate plus LPS treated cells. Individual differences in gene expression levels between the selected animals might contribute to this. However, percent decrease values indicate a slight reduction of IL-8 expression due to butyrate treatment at this time point.

These findings are largely consistent with the mammalian (469) and chicken macrophage literature (90) and indicate that butyrate interferes with the expression of pro-inflammatory cytokines. This is of biological relevance since pre-treatment with butyrate could prevent excessive inflammatory processes from the beginning.

This almost uniform induction effect was particularly visible after 8h, indicating that there might be an application dependent effect. Whether gene expression also decreases with for example late addition of butyrate needs to be clarified in further studies, since this could be therapeutically relevant in ongoing inflammations.

In the mammalian literature, the NF-kB pathway is discussed as possible target of butyrate (116). However, percent decrease values revealed similar induction levels of cytokine expression in the butyrate treated cells due to LPS stimulation. Thus, other mechanisms than the interference with the NF-kB pathway might be responsible for this. Further research is needed in this area, since several mechanisms are involved in the regulation of gene expression due to SCFAs treatment (116).

After 24h of LPS stimulation the inhibitory effect of butyrate in the LPS treated cells was more evident in some cases. These findings indicate a time course dependent regulation of gene expression due to butyrate. This is partly inconsistent with Sunkara et al., who investigated the effect of SCFAs in HD11 cells at gene expression level. In this study the expression levels of IL-1ß and IL-8 after 24h exceeded expression levels measured after 3h. This effect was absent in the case of IL-12 levels (119).

Furthermore, quantitative differences between individual genes were observed in our study. These findings could be due to individual time courses of gene expressions and due to differential regulatory interference between the individual genes (471-473). IL-1ß and TNF-α have been shown to upregulate IL-8 at least in mammals, which is also important for the recruitment of neutrophils (392). In mammals, IL-1ß was able to upregulate IL-1ß transcription by itself (474).

Interestingly, without exception, the expression levels of the cells treated exclusively with LPS were significantly lower after 24h than after 8h. The controls, on the other hand, were largely at the same level at both time points. Considering cells treated with LPS, this is largely consistent with Sunkara et al. who investigated gene expression levels of IL-1ß and IL-8 in HD11 cells 3h and 24h after LPS treatment (119). Autoregulatory processes might be responsible for this. Increasing NO levels (398) in the supernatant, as well as alterations in pH levels (475, 476) have been shown to negatively regulate pro-inflammatory cytokine and iNOS expression and macrophage polarization. Moreover, the time courses of nitric oxide production and iNOS expression differ. After 8h, the NO levels in the supernatant were lower than after 24h in both butyrate-treated cells and cells treated with LPS alone. This is inconsistent with Qui et al. who stimulated the mammalian macrophage cell line RAW 264.7 with LPS (477). In this study, it took more than 3h until iNOS expression was detected. At 6h iNOS was expressed but no NO could be detected in the supernatant. In contrast to our findings, at 24h iNOS expression levels exceeded 6h levels. However, Hussain et al. showed that iNOS activity in cells from various sources differed and iNOS expression in the chicken macrophage cell line MQ-NCSU was lower at 24h compared to 8h (313). Nevertheless, in butyrate plus LPS treated cells the NO levels were constantly lower in this study than in LPS treated cells indicating that NO alone might not be responsible for this downregulation of cytokine expression in LPS treated cells. Furthermore, butyrate appears to be able to potentiate this possibly autoregulatory effect on iNOS expression as revealed by percent decrease values compared to the LPS treated cells.

In conclusion, butyrate significantly decreased the expression levels of several proinflammatory cytokines at 8h and 24h after LPS stimulation. Thus, butyrate may be able to downregulate inflammatory processes by regulating macrophage function, thereby preventing excessive destruction. Moreover, butyrate might also contribute to the acceleration of the resolution of inflammation through influencing autoregulatory anti-inflammatory processes in chicken macrophages.

6.2.5 Outlook

The results of this study suggest the importance of the regulatory function of microbial derived SCFAs on chicken macrophages. Especially butyrate displayed anti-inflammatory properties in several experimental settings and might therefore contribute to tissue homeostasis in the chicken gut as well as systemically. In summary, first steps were taken to better understand this kind of molecular language. Based on these findings, in *vivo* experiments such as feeding trials could be carried out combined with RNA-Seq methods to provide a better insight into the spectrum of regulatory functions of SCFAs on chicken macrophages. Different experimental settings as well as long term studies should be considered hereby to further evaluate the functional versatility and capacity of chicken macrophages to respond to environmental factors. Studies on tissue kinetics and quantitative analyzes could provide a deeper insight into the dynamics of SCFA physiology in the chicken. Further research is needed on a molecular level to identify the exact mechanisms of action of SCFAs in this species.

7 SUMMARY

The original definition of immunology as “science of self non-self discrimination” has been challenged in the last 30 years. Recent research has made it clear that a mutual communication between microbiota and host is necessary to keep a balanced immune system and therefore to maintain internal homeostasis.

Across species, the microbiota seems to influence the immune system at every stage of life.

SCFAs are microbially derived metabolites and especially butyrate is widely used as a feed additive in poultry industry. In humans as well as in chickens, short-chain fatty acids are primarily produced in the intestines. Mainly butyrate has been demonstrated to exert immune-modulating effects.

Macrophages are an essential component of the innate immune system and exhibit a high degree of versatility and plasticity. Besides keeping the body´s internal homeostasis, they play an important role in triggering, maintaining and resolving inflammatory processes. The functional repertoire of these cells covers the spectrum from antimicrobial to tolerogenic properties. In mammals, most macrophages are strategically situated in the intestinal tissue. There, they are confronted with a large number of foreign antigens and yet exhibit a rather hyporesponsive profile. Regulation of macrophage function is therefore a key component in the regulation of inflammatory processes. Only a few studies have examined the effects of SCFAs on chicken macrophages. Macrophages release nitric oxide as part of a defense reaction. Quantification of NO using Griess assay represents a well-established readout system to study macrophage function.

In a first set of experiments, the chicken macrophage cell line HD11 was stimulated with LPS in the absence or presence of SCFAs. In particular butyrate inhibited LPS induced nitric oxide production. To a minor extend, propionate also displayed inhibitory effects. Acetate showed a slight inhibitory effect at best.

In addition, butyrate was also tested on a mammalian macrophage cell line. Confirming the results from the mammalian literature, the inhibitory influence of butyrate treatment on NO production was also evident in RAW 264.7 cells.

Since studies with immortalized cell lines only partially reflect the situation in living animals, SCFAs were tested on primary cells derived from different tissue sources. Macrophages derived from monocytes, bone marrow and spleen were isolated, cultured and stimulated with LPS in the absence or presence of SCFAs.

Butyrate displayed the strongest suppressive effect on the NO production in all cell culture systems. To test the effect of butyrate on tissue-resident macrophages from the intestine, these cells were isolated from the ileum and cecum and subsequently cultured. In contrast to their human counterpart, chicken intestinal macrophages responded to the LPS stimulus by producing NO. Treatment with butyrate inhibited the LPS induced NO production in both ileal and cecal derived macrophages. These findings suggest that butyrate plays an integral part in keeping the intestinal homeostasis in the chicken gut.

Cytotoxic effects due to butyrate treatment have been reported in the literature. To test primary macrophage functions after SCFAs and LPS treatment, two follow-up experiments were performed. After the initial experiment, cells were further cultured for 7 to 10 days in the absence of SCFAs and LPS. Prior to LPS re-stimulation the cell culture supernatant was tested for NO production. NO levels were comparable to the completely untreated cells in the first experiment. Untreated controls from the first experiment were included in this follow-up study. Regardless of the tissue origin primary cells produced NO to LPS re-stimulation. Compared to the initial experiment enhanced as well as diminished NO levels were observed. Mainly macrophages derived from spleen responded with enhanced NO levels. In some cases, suppressive effects due to SCFAs treatment were still evident.

These results could be relevant for the additive use of SCFAs in poultry production. With regard to potential therapeutic use, further gene expression studies are needed to understand the full spectrum of action of SCFAs.

Macrophages use signaling molecules to communicate with other cells. To examine the effects of butyrate on cytokine levels, macrophages derived from spleen were treated with LPS and butyrate. Since chickens lack lymph nodes, the spleen resembles a very important secondary lymphoid organ. After LPS stimulation in the presence or absence of butyrate, butyrate-treated cells displayed lower expression levels at both time points. These findings also highlight the immune-regulatory functions of butyrate in the chicken.

The results of this study show that bacterial metabolites can have widespread effects at both local and systemic levels. A deeper understanding of these signaling pathways is therefore absolutely important.

This study took first exploratory steps towards a better understanding of the host-microbiota relationship in chickens. To decipher this complex language between those two kingdoms, further gene expression-based studies that also consider long-term effects are required.

8 ZUSAMMENFASSUNG

Die ursprüngliche Definition der Immunologie als „Wissenschaft der Selbst-Nicht-Selbst-Diskriminierung“ wurde in den letzten 30 Jahren in Frage gestellt. Neuere Forschungen haben deutlich gemacht, dass eine gegenseitige Kommunikation zwischen Mikrobiota und Wirt notwendig ist, um ein ausgeglichenes Immunsystem und damit die innere Homöostase aufrecht zu erhalten.

Speziesübergreifend scheint die Mikrobiota das Immunsystem in jeder Lebensphase zu beeinflussen.

SCFAs sind mikrobiell gewonnene Metaboliten und insbesondere Butyrat wird häufig als Futterzusatzstoff in der Geflügelindustrie verwendet. Sowohl beim Menschen als auch beim Huhn werden kurzkettige Fettsäuren hauptsächlich im Darm produziert. Vor allem Butyrat hat nachweislich eine immunmodulierende Wirkung.

Makrophagen sind ein wesentlicher Bestandteil des angeborenen Immunsystems und weisen ein hohes Maß an Vielseitigkeit und Plastizität auf. Neben der Aufrechterhaltung der körpereigenen Homöostase spielen sie eine wichtige Rolle bei der Auslösung, Aufrechterhaltung und Lösung von Entzündungsprozessen. Das Funktionsrepertoire dieser Zellen umfasst das Spektrum von antimikrobiellen bis tolerogenen Eigenschaften. Bei Säugetieren sind die meisten Makrophagen strategisch im Darmgewebe lokalisiert. Dort werden sie mit einer Vielzahl fremder Antigene konfrontiert und weisen dennoch ein eher hyporesponsives Profil auf. Die Regulierung der Makrophagenfunktion ist daher eine Schlüsselkomponente bei der Regulierung entzündlicher Prozesse. Nur wenige Studien haben die Auswirkungen von SCFAs auf Hühnermakrophagen untersucht. Makrophagen setzen im Rahmen einer Abwehrreaktion Stickstoffmonoxid frei. Die Quantifizierung von NO mithilfe des Griess-Assays stellt ein gut etabliertes Auslesesystem zur Untersuchung der Makrophagenfunktion dar.

In einer ersten Versuchsreihe wurde die Hühnermakrophagenzelllinie HD11 mit LPS in Abwesenheit oder Gegenwart von SCFAs stimuliert. Insbesondere Butyrat hemmte die durch LPS induzierte Stickoxidproduktion. In geringem Maße zeigte Propionat auch hemmende Wirkungen. Acetat zeigte bestenfalls eine leichte Hemmwirkung.

Darüber hinaus wurde Butyrat auch an einer Makrophagenzelllinie von Säugetieren getestet. In Bestätigung der Ergebnisse aus der Säugetierliteratur war der hemmende Einfluss der Butyratbehandlung auf die NO-Produktion auch in RAW264.7-Zellen offensichtlich.

Da Studien mit immortalisierten Zelllinien die Situation bei lebenden Tieren nur teilweise widerspiegeln, wurden SCFAs an Primärzellen verschiedener Gewebequellen getestet. Aus Monozyten, Knochenmark und Milz stammende Makrophagen wurden isoliert, kultiviert und mit LPS in Abwesenheit oder Anwesenheit von SCFAs stimuliert.

Butyrat zeigte in allen Zellkultursystemen die stärkste unterdrückende Wirkung auf die NO-Produktion. Um die Wirkung von Butyrat auf geweberesidente Makrophagen aus dem Darm zu testen, wurden diese Zellen aus dem Ileum und Blinddarm isoliert und anschließend kultiviert. Im Gegensatz zu ihrem menschlichen Gegenstück reagierten Darmmakrophagen von Hühnern auf den LPS-Stimulus mit der Produktion von NO. Die Behandlung mit Butyrat hemmte die LPS-induzierte NO-Produktion sowohl in ilealen als auch in caecalen Makrophagen. Diese Ergebnisse legen nahe, dass Butyrat eine wesentliche Rolle bei der Aufrechterhaltung der Darmhomöostase im Hühnerdarm spielt.

In der Literatur wurde über zytotoxische Wirkungen aufgrund der Butyratbehandlung berichtet. Um die Funktionen primärer Makrophagen nach SCFAs- und LPS-Behandlung zu testen, wurden zwei Folgeexperimente durchgeführt. Die Zellen wurden 7 bis 10 Tage lang in Abwesenheit von SCFAs und LPS weiter kultiviert. Vor der LPS-Restimulation wurde der Zellkulturüberstand auf NO-Produktion getestet. Die NO-Werte waren vergleichbar mit denen der völlig unbehandelten Zellen im ersten Experiment. Unbehandelte Kontrollen aus dem ersten Experiment wurden in diese Folgestudie einbezogen. Unabhängig von der Gewebeherkunft produzierten Primärzellen bei LPS-Restimulation NO. Im Vergleich zum ersten Experiment wurden sowohl erhöhte als auch verringerte NO-Werte beobachtet. Hauptsächlich aus der Milz stammende Makrophagen reagierten teilweise mit erhöhten NO-Werten. In einigen Fällen waren noch immer unterdrückende Wirkungen aufgrund der SCFA-Behandlung erkennbar.

Diese Ergebnisse könnten für den additiven Einsatz von SCFAs in der Geflügelproduktion relevant sein. Im Hinblick auf einen möglichen therapeutischen Einsatz sind weitere Genexpressionsstudien erforderlich, um das gesamte Wirkungsspektrum von SCFAs zu verstehen.

Makrophagen nutzen Signalmoleküle, um mit anderen Zellen zu kommunizieren. Um die Wirkung von Butyrat auf den Zytokinspiegel zu untersuchen, wurden aus der Milz stammende Makrophagen mit LPS und Butyrat behandelt. Da Hühnern Lymphknoten fehlen, ähnelt die Milz einem sehr wichtigen sekundären Lymphorgan. Nach LPS-Stimulation in Gegenwart oder Abwesenheit von Butyrat zeigten mit Butyrat behandelte Zellen ausnahmslos geringere Expressionsniveaus. Diese Ergebnisse unterstreichen auch die immunregulatorischen Funktionen von Butyrat.

Die Ergebnisse dieser Studie zeigen, dass bakterielle Metaboliten sowohl auf lokaler als auch auf systemischer Ebene weitreichende Auswirkungen haben können. Ein tieferes Verständnis dieser Signalwege ist daher unbedingt wichtig.

Diese Studie unternahm erste explorative Schritte zu einem besseren Verständnis der Wirt-Mikrobiota-Beziehung bei Hühnern. Um diese komplexe Sprache zwischen diesen beiden Königreichen zu entschlüsseln, sind weitere auf der Genexpression basierende Studien erforderlich, die auch langfristige Auswirkungen berücksichtigen.

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