Immunomodulatory effects of resveratrol on human intestinal mast cell signaling in vitro and mast cell associated enteritis and colitis in mice

Dissertation to obtain the doctoral degree of

Natural Sciences (Dr. rer. nat.)

Faculty of Natural Sciences

University of Hohenheim

Institute of Nutritional Medicine & Prevention

submitted by

Sabrina Bilotta

from Göppingen, Germany

2022

This thesis was accepted by the Faculty of Natural Sciences at the University of Hohenheim on November 11, 2022 as “Dissertation for obtaining the doctoral degree of Natural Sciences“ (Dr. rer. nat).

Dean of faculty	Prof. Dr. rer. nat. Uwe Beifuss
Supervisor and 1st reviewer	Prof. Dr. rer. nat. Axel Lorentz
2nd reviewer	Prof. Dr. med. Dr. rer. nat. Sascha Venturelli
Additional examiner & head of committee	Prof. Dr. rer. nat. Jan Frank
Date of the oral examination	February 06, 2023

For 93 mice

and all experimental animals

CONTENT OVERVIEW

MANUSCRIPTS

As first author

Resveratrol Is a Natural Inhibitor of Human Intestinal Mast Cell Activation and Phosphorylation of Mitochondrial ERK1/2 and STAT3

Sabrina Bilotta, Lakshmi Bhargavi Paruchuru, Katharina Feilhauer, Jörg Köninger and Axel Lorentz - published in International Journal of Molecular Sciences, 2021, July 16th

[a] *Bilotta S, Paruchuru LB, Feilhauer K, Köninger J, Lorentz A. Resveratrol Is a Natural Inhibitor of Human Intestinal Mast Cell Activation and Phosphorylation of Mitochondrial ERK1/2 and STAT3. Int J Mol Sci. 2021 Jul 16;22(14):7640. doi: 10.3390/ijms22147640. PMID: 34299258; PMCID: PMC8307672.*

Resveratrol Treatment Prevents Increase of Mast Cells in Both Murine OVA Colitis and IL-10$^{-/-}$ Colitis

Sabrina Bilotta, Julian Arbogast, Nadine Schart, Maurice Frei and Axel Lorentz - published in International Journal of Molecular Sciences, 2022, January 21st

[a] *Bilotta S, Arbogast J, Schart N, Frei M, Lorentz A. Resveratrol Treatment Prevents Increase of Mast Cells in Both Murine OVA Enteritis and IL-10$^{-/-}$ Colitis. Int J Mol Sci. 2022 Jan 21;23(3):1213. doi: 10.3390/ijms23031213. PMID: 35163137.*

As co-author

Resveratrol Attenuates Mast Cell Mediated Allergic Reactions: Potential for Use as a Nutraceutical in Allergic Diseases?

Mehtap Civelek, Sabrina Bilotta and Axel Lorentz – published in Molecular Nutrition & Food Research, 2022, May 22nd

[b] *Civelek M, Bilotta S, Lorentz A. Resveratrol Attenuates Mast Cell Mediated Allergic Reactions: Potential for Use as a Nutraceutical in Allergic Diseases? Mol Nutr Food Res. 2022 May 22:e2200170. doi: 10.1002/mnfr.202200170. Epub ahead of print. PMID: 35598149.*

Further publications produced in the course of the doctoral project

Molecular links between allergy and cancer

Axel Lorentz, Sabrina Bilotta and Mehtap Civelek – published in Trends in Molecular Medicine, 2022, July 4th

[b] *Lorentz A, Bilotta S, Civelek M. Molecular links between allergy and cancer. Trends Mol Med. 2022 Jul 4:S1471-4914(22)00158-7. doi: 10.1016/j.molmed.2022.06.003. Epub ahead of print. PMID: 35794030.*

Sabrina Bilotta contributed to the manuscripts in the following way:

[a] Experimental work; statistics; data interpretation, manuscript conception and preparation as well as figure preparation

[b] Finalization of the manuscript; figure preparation

I hereby confirm the declaration of the candidate's contributions to the dissertation:

Stuttgart, 4.11.22

Place, date

apl. Prof. Dr. Axel Lorentz

ABSTRACT

By releasing their pre-stored or *de novo* synthesized mediators, mast cells (MC) are important immunoregulatory cells responsible for a variety of inflammatory reactions. Although known to be major effector cells in immunoglobuline (Ig) E dependent allergic reactions, MC have been widely shown to play a role in various inflammations of the gut. Diseases of the gastrointestinal tract (GIT) are widespread and multicausal. Those affected suffer from the sometimes severe symptoms and may experience restrictions on their daily life. Even if conventional medication is applied routinely, aim of the past and current research is to establish supportive and/or alternative medication that is based on natural substances. These may be on the basis of small natural components like resveratrol, a stilbene mostly found in grapes. Numerous positive properties are attributed to resveratrol. These are anti-inflammatory, anti-cancerogenic, anti-oxidative, as well as neuroprotective effects. The use of substances of natural origin as so-called nutraceuticals can help to increase the acceptance of medication by those affected, but also to reduce and overcome the side effects associated with conventional treatment.

Effects of resveratrol were examined on the reactivity of MC isolated from patients' tissue undergoing bowel resection. The results of this work show that resveratrol exhibited potent inhibitory effects on high affinity IgE receptor mediated activation of MC, strongly inhibiting not only MC degranulation, but also gene expression of the pro-inflammatory cytokines C-X-C motif chemokine ligand (*CXCL*) *8*, C-C motif chemokine ligand (*CCL*) *2*, *CCL3*, *CCL4* and tumor necrosis factor (*TNF*-) *α*. Ultimately, the intracellular signaling cascade triggered during MC activation via IgE receptor leads to mediator release. Following IgE receptor mediated activation, phosphorylation of signaling molecules like extracellular signal-regulated kinase (ERK) 1/2 and signal transducer and activator of transcription (STAT) 3, occurs. ERK1/2 was found to be responsible for phosphorylation of mitochondrial STAT3, which contributes significantly to MC degranulation. Treatment with resveratrol was able to inhibit the phosphorylation of STAT3 by more than 50 % and that of ERK1/2 by almost 100 %. Furthermore, the experiments performed succeeded in isolating the mitochondrial fraction from relatively low human intestinal MC (hiMC) numbers. Also, in this fraction we could detect phosphorylation of STAT3 and ERK1/2 after MC activation, which was reduced after treatment with resveratrol.

Having shown the strong inhibitory effects *in vitro*, we set out to examine immunomodulatory effects of resveratrol *in vivo*. Presence and activity of MC are closely related to intestinal inflammations in consequence of food allergy (FA) and inflammatory bowel disease (IBD). In mice, FA can be studied using the ovalbumin (OVA)-induced allergic enteritis model and colitis can be studied using the IL-10 knockout ($^{-/-}$) mice, which develop a spontaneous form of chronic

colitis. We could show that the oral application of resveratrol inhibited the increase of MC numbers in the colon and duodenum of affected animals in both experimental settings. Less pronounced but still visible effects of resveratrol administration were observed in the colon with regard to epithelial damage, cell infiltration and reduction of goblet cell numbers. In all cases, based on a scoring system, the damage decreased to the level of the corresponding controls receiving no additive and in which no allergic enteritis was induced or nor colitis developed. Overall, allergic enteritis resulted in a weaker symptomatology, and IL-10$^{-/-}$ animals showed a delayed appearance of the typical symptoms.

The results of this thesis show a strong inhibitory effect of resveratrol on hiMC. This could be detected for mediator release as well as on gene expression levels and in the phosphorylation of the signaling molecules ERK1/2 and STAT3, which we could also identify in the mitochondria of hiMC. We observed positive influences on MC-associated parameters in the OVA enteritis and IL-10$^{-/-}$ colitis mouse models. With regard to its use as nutraceutical, resveratrol could therefore come more of a focus in the future.

ZUSAMMENFASSUNG

Mastzellen sind wichtige immunregulatorische Zellen, die durch Freisetzung ihrer in den Granula gespeicherten oder der *de novo* synthetisierten Mediatoren für eine Vielzahl von Entzündungsreaktionen verantwortlich sind. Mastzellen, die in erster Linie als Haupteffektorzellen bei von Immunoglobulin (Ig) E abhängigen allergischen Reaktionen bekannt sind, spielen auch bei diversen Entzündungen des Darmes eine Rolle. Erkrankungen des Gastrointestinaltraktes sind weit verbreitet und multikausal. Betroffene leiden aber immer mit unter der teils stark auftretenden Symptomatik und erfahren Einschränkungen im alltäglichen Leben. Auch wenn die herkömmliche Medikation zur Behandlung dieser Störungen routinemäßig Anwendung findet, ist es Ziel der Wissenschaft, eine unterstützende sowie Alternativmedikation zu entwickeln, die auf natürlichen Ausgangssubstanzen, wie z.B. dem in Trauben vorkommenden Stilben Resveratrol, basiert. Resveratrol werden zahlreiche positive Eigenschaften zugeschrieben. Darunter fallen anti-inflammatorische, anti-kanzerogene, antioxidative, als auch neuroprotektive Eigenschaften. Ein Einsatz von Substanzen natürlichen Ursprungs als sogenanntes Nutraceutical kann einerseits dazu dienen, die Akzeptanz der Medikation bei Betroffenen zu erhöhen, aber auch, die mit der herkömmlichen Behandlungsmethode verbundenen, Nebenwirkungen zu mindern und zu überwinden.

Effekte von Resveratrol wurden auf die Reaktivität von Mastzellen, isoliert aus humanen intestinalen Resektionspräparaten (hiMC), geprüft. Die Ergebnisse dieser Arbeit verdeutlichen, dass das Stilben starke inhibitorische Wirkung auf IgE-Rezeptor vermittelte Aktivierung von Mastzellen zeigt und dabei nicht nur die Mastzelldegranulation, sondern auch die Genexpression der pro-inflammatorischen Zytokine *C-X-C motif chemokine ligand* (*CXCL*) *8, C-C motif chemokine ligand* (*CCL*) *2, CCL3, CCL4* und *tumor necrosis factor (TNF-*) *α* stark hemmt. Die, im Zuge der Mastzellaktivierung via IgE-Rezeptor, ausgelöste intrazelluläre Signalkaskade führt letztlich zur Mediatorausschüttung. Im Verlauf der Signalkaskade kommt es zur Phosphorylierung, d.h. der Aktivierung der Signalmoleküle *extracellular signal-regulated kinase* (ERK) 1/2 und *signal transducer and activator of transcription* (STAT) 3. Dabei ist ERK1/2 für die Phosphorylierung von mitochondrialem STAT3 verantwortlich, welches maßgeblich zur Mastzelldegranulation beiträgt. Die Behandlung mit Resveratrol führte dabei zur Hemmung der Phosphorylierung von STAT3 um mehr als 50 %, sowie die von ERK1/2 um fast 100 %. Des Weiteren gelang es uns, die mitochondriale Fraktion aus relativ geringen Mengen humaner intestinaler Mastzellen (hiMC) zu isolieren. Auch in dieser Fraktion konnten wir die Phosphorylierung von ERK1/2 und STAT3 nach Mastzellaktivierung detektieren, die nach einer Behandlung mit Resveratrol ebenfalls verringert war.

Nachdem die stark hemmende Wirkung von Resveratrol *in vitro* gezeigt wurde, sollten die immunmodulatorischen Wirkungen auch *in vivo* überprüft werden. Das Vorhandensein und die Aktivität von MC stehen in engem Zusammenhang mit Entzündungen des Darms als Ursache von Nahrungsmittelallergien sowie chronisch-entzündlicher Darmerkrankungen (CED). Im Mausmodell kann man Nahrungsmittelallergie mit Hilfe einer Ovalbumin (OVA)-induzierten allergischen Enteritis und CED mit Hilfe der IL-10 knockout ($^{-/-}$) Maus, bei der es zu einer spontan auftretenden chronischen Colitis kommt, experimentell untersuchen. Wir konnten zeigen, dass die orale Gabe von Resveratrol bei betroffenen Tieren im Allergiemodell (OVA) als auch im Modell der murinen Colitis (IL-10$^{-/-}$) zu einer Hemmung des Anstiegs der Mastzellanzahl im Gewebe von Colon und Duodenum betroffener Tiere führte. Weniger stark ausgeprägte, aber dennoch sichtbare Effekte bei einer Resveratrol-Gabe konnten im Colon im Hinblick auf Epithelschädigung und bei der IL-10$^{-/-}$ Colitis auch im Hinblick auf Zellinfiltration sowie Reduktion der Anzahl an Gobletzellen beobachtet werden. In allen Fällen verringerten sich die Schädigungen auf das Level der jeweiligen Kontrolltiere ohne allergische Enteritis bzw. Colitis. Insgesamt kam es bei der allergischen Enteritis zu einer schwächer ausgeprägten Symptomatik, bei IL-10$^{-/-}$ Tieren kam es insgesamt zu einem verzögerten Auftreten der typischen Symptomatiken.

Die Ergebnisse dieser Promotionsarbeit zeigen eine stark inhibitorische Wirkung von Resveratrol auf hiMC. Diese ließen sich sowohl bei der Mediatorfreisetzung als auch auf Genexpressionslevel und in der Phosphorylierung der Signalmoleküle ERK1/2 und STAT3 zeigen, bei denen es gelungen ist, sie auch in den Mitochondrien von hiMC nachzuweisen. Auch im Mausmodell bei der Untersuchung einer allergisch induzierten Enteritis sowie einer murinen Colitis aufgrund eines IL-10$^{-/-}$ konnten wir positive Einflüsse auf Mastzell-assoziierte Parameter beobachten. Im Hinblick auf die Verwendung als Nutraceutical könnte Resveratrol deshalb zukünftig mehr in den Fokus rücken.

INTRODUCTION

1. Mast cells

Mast cells (MC) are the key players in the development of type-I mediated immune reactions induced by immunoglobulin (Ig) E. These immune responses are also called early phase reactions. In 1878, Paul Ehrlich described these cells with plenty of embedded granules and making them look like a “well-fed” cell as “Mastzelle” [1]. In homeostasis, MC have the physiological functions of angiogenesis, interaction with the nervous system, blood flow regulation, tissue repair and interactions with the immune system [2, 3]. Due to their localization at sites of potential antigen entry, MC display an important immunoregulatory role. After maturation from CD34+/CD117+ progenitor cells and migration into connective and vascularized tissues [4-6], differentiation under the influence of several growth factors takes place. The most important one is stem cell factor (SCF) together with Interleukin (IL) 4 [7] in humans or murine IL-3 in mouse tissue [7, 8].

MC are primarily located in tissues with sites of potential antigen entry such as skin, lung and the gastrointestinal tract of the body [9-11]. In the gut, MC are mainly localized in mucosal lamina propria, representing 2-3 % of all cells [11], about 1 % of all cells are found in the submucosal parts [12]. Additional presence is described in brain tissue by Traina [13], showing their role and influencing effects on the microbiota-gut-brain axis. Low numbers are found in kidney and pancreas under physiological state but enhanced in pathological conditions like diabetes, glomerulonephritis or pancreatitis [3].

MC are main players in inflammatory and immediate allergic reactions by releasing their potent mediators after immunologically activation via different receptors expressed on MC surface. The most prominent activation signal of MC and initiator of early phase reactions is the crosslinking of the high-affinity FcεRI receptor after an initial sensitization phase (Figure 1). After allergen contact, antigen-presenting cells like dendritic cells present parts of it via their major-histocompatibility complex II to T cell receptor of T cells. These T cells in turn produce and secrete IL-4, which on the one hand is needed to maintain IL-4 rich milieu important for T helper (Th) 2-cells’ function and on the other hand, together with IL-13 stimulates B cells to undergo heavy chain class-switching from IgM to IgE. This is supported by the ligation of CD40 with CD40 ligand and CD80/CD86 with CD28 [14]. Secreted IgE antibodies then bind to the high affinity FcεRI receptors on MC, making them sensitive to this respective allergen [15, 16]. Re-contact with the same allergen, which is then bound to membrane localized IgE, leads to crosslinking of the receptor by linkage of at least two of these molecules [17]. The γ-subunit of

FcεRI initiates the downstream signaling cascades leading to release of numerous mediators [18]. This so-called type I hypersensitivity reaction together with other activation routes results in release of pre-stored proteases (tryptase, chymase), biogenic amines (histamine) or enzymes like β-hexosaminidase as well as *de novo* synthesized cytokines/chemokines, lipid mediators (prostaglandins, leukotrienes) derived from arachidonic acid [14, 19, 20]. These mediators recruit other cells of the immune system to sites of inflammation and are responsible for maintenance of inflammatory allergic reactions that, if becoming chronic, manifest in variuos diseases like asthma, allergic rhinitis or atopic dermatitis [14]. Further, MC are involved in development of autoimmune disorders, atherosclerosis or mastocytosis [21].

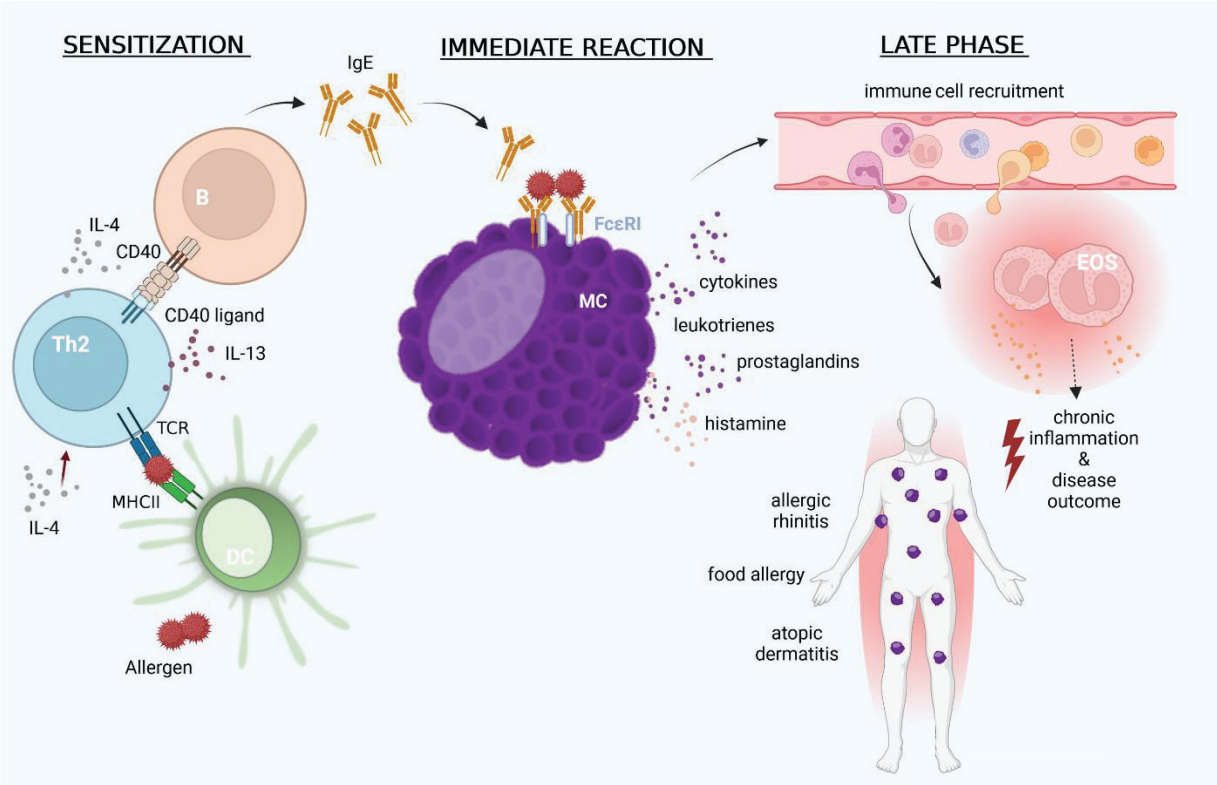

Figure 1 - Overview of hypersensitivity reaction and mast cell associated diseases. Allergens are up taken by dendritic cells (DC) which then present parts of it to T-cell receptors (TCR) on T-cells via their own major histocompatibility complex (MHC) II receptor. The sensitization phase is further marked by B cells undergoing heavy-chain class switching from immunoglobulin (Ig) M to IgE under the influence of Interleukin (IL) 4 and IL-13. Immediate reactions are characterized by binding of IgE to the high affinity receptor FcεRI on mast cells (MC). Crosslinking of FcεRI further induces the release of several mediators. In the late phase, these mediators are responsible for the recruitment of other immune cells, one of them being eosinophils (EOS). Recruitment and activity of these cells may lead to chronic inflammations in different parts of the body, provoking serious disease outcome of several kind (adapted and modified from own work [22, 23]; created with BioRender.com).

2. Mast cell signaling

MC activation and degranulation depends on several different activation signals. As already described, the most common activation pathway is crosslinking of FcεRI receptor. This receptor is built out of four subunits, namely an IgE-binding α chain, a membrane spanning β chain and a disulfide-linked homodimer of two γ chains [5, 24, 25]. Src kinases Lyn and Syk are crucial for initiation of the degranulation phase as the high affinity receptor lacks an intrinsic tyrosine kinase activity [24]. Linkers for activation of T cells (LAT) get phosphorylated by Syk and subsequent recruitment of phospholipase Cγ (PLCγ) drives hydroxylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and activates membrane-bound diacylglycerol (DAG). Interaction of the two second messengers IP_3 and DAG, but primarily IP_3, provokes calcium (Ca^{2+}) influx from the endoplasmatic reticulum (ER) leading to e.g. the release of histamine. Activation of LAT is further responsible for activity of mitogen activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) or p38 [26] dependent on Ras in the presence of GDP/GTP [27]. Ca^{2+} influx induced by IP_3 together with DAG stimulates protein kinase C (PKC) and its activity on MAPK, which then are responsible for translocation of nuclear factor kappa B (NF-κB) and other transcription factors into the nucleus and the final induction of gene expression of several cytokines and chemokines [27].

Mas-related G-protein coupled receptor member (MRGPR) X2 (ligands; e.g. Substance P, C48/80) [28] represents an IgE-independent way of MC induction. Same as for IgE receptor, it is responsible for PLCγ activation. Besides MRGPRX2, there are some more IgE-independent activation signals leading to degranulation processes and/or *de novo* synthesis and release of mediators in MC. Amongst these, IL-33 by binding to its membrane-bound interleukin 1 receptor-like 1 (ST2) [29] or bacterial components such as lipopolysaccharides (LPS) onto toll-like receptors (TLR) induce myeloid differentiation primary response (MyD) 88 and subsequent activity of IL-1 receptor-associated kinase (IRAK). IRAK induces activation of MAP kinases ERK, p38 or JNK as well as NF-κB translocation [24, 30]. Further, MC express receptor for advanced glycation end (RAGE) offering binding sites for ligands such as advanced glycation end products (AGEs) [31]. Same as for MyD88-IRAK interaction, RAGE induction leads to transcription of cytokines/chemokines via NF-κB or MAPK [32]. One of the most important receptors expressed on MC, besides FcεRI, is CD117 receptor (c-Kit, Kit). CD117 acts as binding site for SCF, the MC growth factor responsible for development, survival and final maturation together with IL-4 in human MC [7]. Both, Kit as well as FcεRI are responsible for phosphorylation of non–T-cell activation linker (NTAL), representing a pivotal link in the degranulation process induced via these two receptors [33]. Figure 2 gives an

overview over some of the possible activation routes of MC and the induced downstream signaling cascades leading to degranulation.

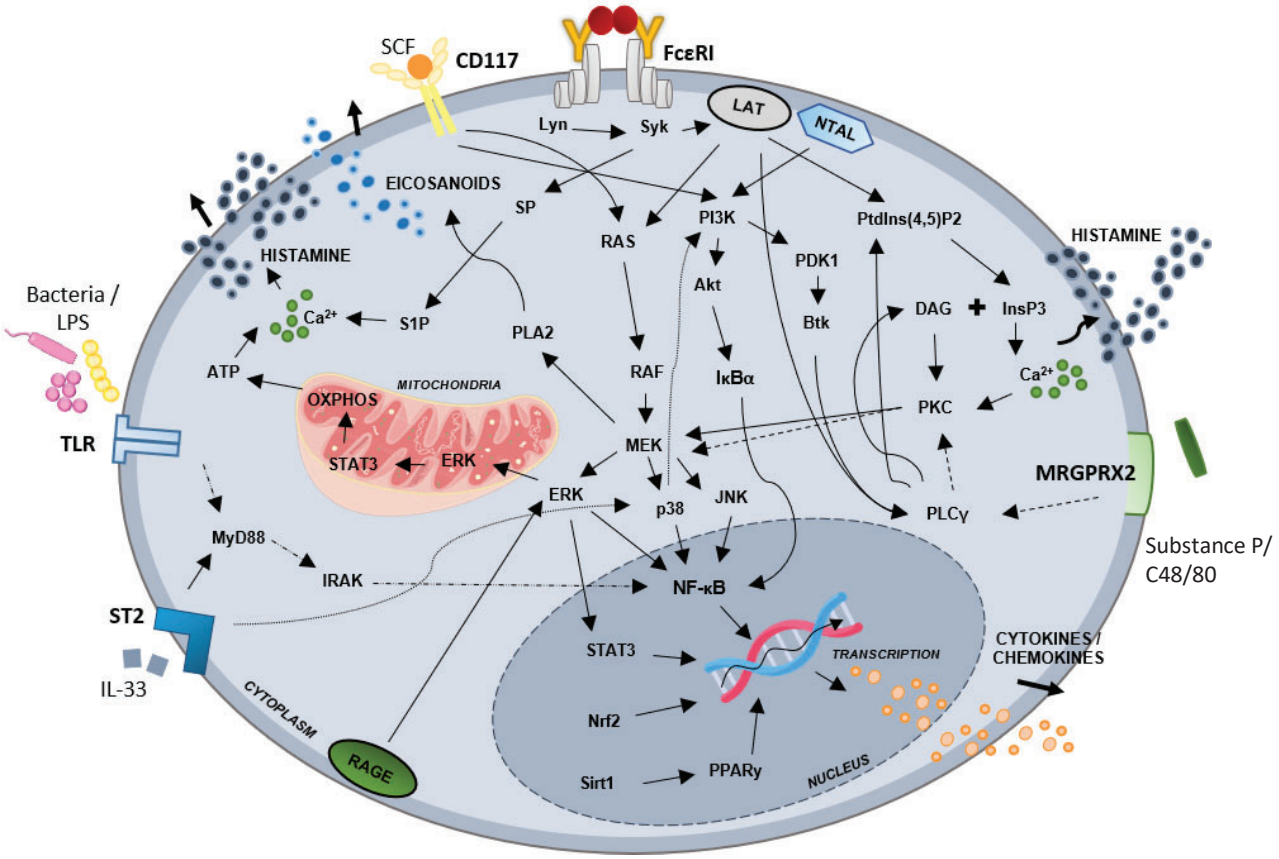

Figure 2 – **Signaling pathways in mast cells**. MC are activated via several routes including amongst others CD117, FcεRI, toll-like (TL), ST2 and MRGPRX2 receptors after binding of their effectors, namely SCF, IgE, bacterial components like LPS, IL-33 and substance P or C48/80, respectively. Induced signaling cascades lead to release of pre-stored mediators like histamine as well as *de novo* production of cytokines and chemokines (adapted & modified from own work [22]).

3. Role of mitochondrial components in mast cell signaling

MC activation initiates various downstream signaling pathways. These pathways, leading to degranulation processes and production of newly synthesized mediators, include the presence of intracellular Ca^{2+} levels [34]. After being uptaken into the mitochondrial matrix, Ca^{2+} influences the adenosine triphosphate (ATP) production [35] and is responsible for translocation of mitochondria to exocytosis sites [36]. Energy as ATP was shown to be crucial for MC exocytosis [37]. ATP is generated from oxidative phosphorylation (OXPHOS) in

mitochondria of cells and this mitochondrial ATP, but not glycolytic ATP, was shown to cause degranulation of MC [37]. Not only ATP, but also the mitochondrial signaling molecule signal transducer and activator of transcription (STAT) 3 is involved in energy production leading to MC degranulation [37, 38]. These observations were extended by the finding that ERK is responsible for the phosphorylation of STAT3 on its serine residue 727 and subsequent OXPHOS induction in mitochondria [37]. Microphthalmia transcription factor (MITF), besides its canonical role as a transcription factor same as for STAT3, was found to interact with pyruvate dehydrogenase (PDH), a regulator of ATP production in mitochondria [39] and experiments already demonstrated a strong interaction between STAT3, its main protein inhibitor of activated STAT3 (PIAS3) and MITF [40]. These observations show that mitochondrial signaling molecules are essential for functionality of MC.

4. Allergy

"*What is food to one, to another is rank poison*" (Titus Lucretius Caro) – this quote can be found in a poem from more than 2000 years ago and shows that hypersensitive reactions to substances were a matter back then, implied through former descriptions in Chinese literature about 2800 years BC [41, 42]. Even Hippocrates made observations of cheese idiosyncrasy, the oldest allergological term, which was first used in the ancient world [41, 43]. It seems that decades ago, people were aware of allergic reactions without having a clear definition of the observed symptoms. For the first time defined through *von Pirquet* in 1906 [42, 44], the term allergy nowadays is defined as a hypersensitivity reaction which is indicated by specific immunologic mechanisms [14] and includes allergic disorders which can be divided into IgE-dependent and IgE-independent reactions [45]. One can observe an abnormal immune response directed against normally harmless substances from the environment and infectious organisms [14].

For years, there has been an increase in diseases associated with allergy [46]. Even though being a small part in all occurring forms of allergy, food allergies with an amount of 4,7 % up to 10 % [47, 48] show increase in prevalence [49] not only in western countries but also in developing areas such as Asia [48, 50]. This is due to industrialization and adaption to western lifestyles [51] and therefore being a major public health problem in industrialized regions of the world [52]. People with food allergies suffer from an extenuated quality of life [53, 54] due to limitations in lifestyle and daily habits.

Talking about hypersensitivity in general, makes it necessary to differentiate between different types classified by Coombs and Gell in 1963 [55, 56]. Hypersensitivity is thereby classified into

immediate type-I (IgE-mediated hypersensitivity), type-II (antibody-mediated cytotoxic hypersensitivity, e.g. IgG-mediated), type-III (immune complex-mediated hypersensitivity) or into a delayed type-IV (cell-mediated) hypersensitivity.

Food allergy (FA) is mostly induced via type-I reactions, representing a non-toxic, immunological adverse reaction to one or more food components such as those of eggs, nuts, soy, wheat, milk or fish [57, 58]. An important impact for food or food component tolerance is an intact gastrointestinal barrier [59, 60]. Loss of barrier integrity, together with breakdown of oral tolerance towards ingested food particles, may lead to FA [58, 59], as antigens are able to permeate the gastrointestinal barrier in an easier way. Nonetheless, also genetics or environmental factors are discussed to be involved in FA development [59]. Commonly, food components are recognized as harmless (oral tolerance) and the subsequent immune reaction after food ingestion is displayed by antigen presention via $CD103^+$ dendritic cells which then induce T reg cells in lymph nodes under the presence of IL-10 and transforming growth factor β [57- 59]. In FA, T reg cell production is inhibited whilst Th2 cells are induced via dendritic cells and driven to promote IgE class switching of B cells in the course of sensitization [59]. After a new antigen contact, IgE binds to the high affinity receptor of MC inducing the respective activation signals (Figure 1 and Figure 2).

Manifestation of FA is reported as oral allergy syndrome [61], eosinophilic oesophagitis, gastritis and enteritis as well as inflammation of the small intestine, colon and rectum [62]. Symptoms accompanied with allergic enteritis are irritability, abdominal pain, flatulence, colic and diarrhea. Stools may contain blood as well as mucus. On a microscopical level, inflammation, bleeding, extravasation, erythema and swelling together with the typical infiltration of eosinophils to the respective inflamation sites were found [62].

5. Inflammatory bowel disease (IBD)

Inflammations of the gastrointestinal tract (GIT) and impairment of the intestinal barrier leading to chronic uncontrollable inflammatory reactions are called Inflammatory bowel disease (IBD) [63, 64]. These inflammatory reactions may be as extensive, as to there is an extraintestinal manifestation which affect joints, skin or other organs like liver or pancreas [64, 65]. The most common disorders in IBD are ulcerative colitis (UC) and Crohn's disease (CD) [11]. For years, IBD is associated with microbiota and gut dysbiosis [66, 67] as well as a translocation of bacteria across the intestinal barrier [68].

The first reports of UC date back to ancient times. A description of UC symptoms as we know them to date was first given by Sir Samuel Wilks in 1859, as today it is said that this might has been more a description of CD rather than UC. In 1888, after a report describing UC cases with no other known causes, the term “ulcerative colitis” was used in the general medical vocabulary [69]. The clinical picture of CD was separated from that of UC by Crohn in 1932, although the first complete description of CD goes back to Morgagni back in 1761 [69].

Differentiation between UC and CD is made due to the manifestation of the respective clinical symptoms (Figure 3). While CD affects all parts of GIT including the mouth and anus, UC is limited to parts of the colon [70]. It is discussed that the manifestation of IBD is a result of environmental, microbial and immune-mediated interactions based on genetically susceptible hosts, which are further reinforced by several extern risk factors as smoking, antibiotic use or diet summarized in Figure 3 [71, 72]. Prevalence of IBD increased throughout the past decades in western countries, incidence shows increasing tendency in new industrialized countries [71, 73, 74].

There are a plenty of animal and murine models available to study IBD as reviewed elsewhere [75]. These models are classified into five different groups: congenic, chemically induced, genetically engineered, cell transfer based or spontaneous models. A prominent and frequently used model based on genetic modification is the IL-10 knockout (IL-$10^{-/-}$) mouse, which develops a spontaneous form of chronic colitis after a short lifetime due to the missing anti-inflammatory cytokine IL-10 [76]. Commonly used models are those of chemically induced trinitrobenzene sulfonic acid (TNBS) and dextran sulfate sodium (DSS) acute colitis, as their effects and appearance of disease patterns can be achieved quickly and inexpensively [77, 78]. Each model available got its advantages and disadvantages and respective models should be chosen due to the scientific questions to be addressed [79].

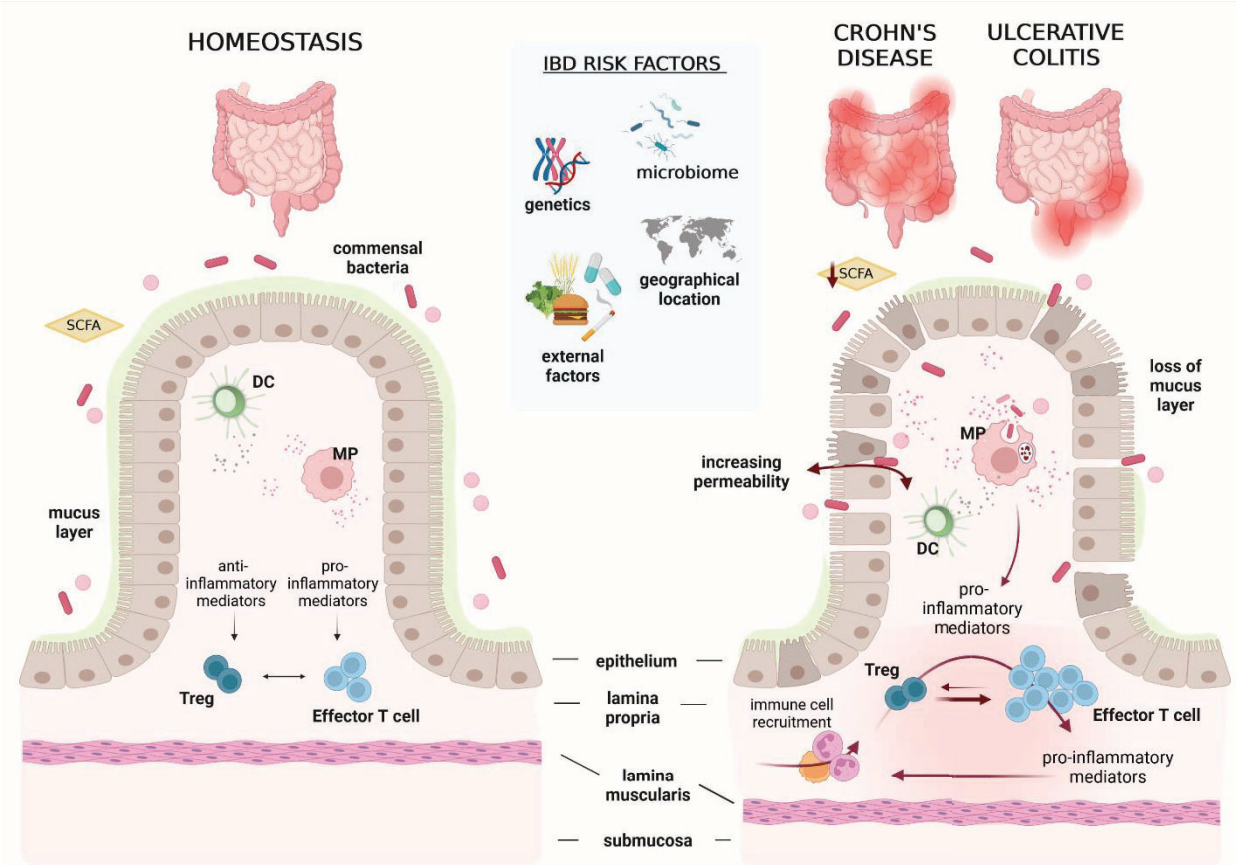

Figure 3 – Comparison of homeostatic and inflammatory state of the gut in IBD. In homeostasis, an intact mucus layer ensures that commensal bacteria cannot penetrate through the epithelial layer. Anti-microbial peptides maintain the mucus layer together with short-chain fatty acids (SCFAs) produced by commensal bacteria. These, as well as anti- and pro-inflammatory mediators from DC and macrophages (MP) ensure a balanced presence of regulatory T cells (Treg) and effector T cells. IBD risk factors induce dysbiosis of the commensal microbiota. Consequently, fewer protective SCFAs are produced, the integrity of the epithelial layer is lost, and the mucosal layer recedes. Bacterial invasion causes local immune responses and attraction of more immune cells to affected sites, consequently the production of pro-inflammatory cytokines is also increased. This in turn disturbs the balance of Treg & effector T cells, leading to the increase of the latter. A vicious circle develops and a chronic inflammatory state occurs (adapted and modified after [80]; created with BioRender.com).

6. Treatment of inflammatory disorders like allergy or IBD

The “hygiene hypothesis”, first introduced by Strachan back in 1989 [81], was proposed as one of the main reasons of allergic and other and autoimmune disease manifestation [71] with a synchron decrease of infectious disorders [82, 83]. Simultaneously, life expectancy increased or is predicted to further do so [84, 85], and occurrence of allergies or IBD need to be treated due to limitations in daily life caused by a wide range of symptoms. Treatment of both, allergy

and IBD, is well-established for years and conventional pharmaceutical medication [70, 86, 87] ameliorates the symptoms and helps the patients in maintaining a better life quality. Nonetheless, there are reports of negative side effects such as nausea or vomiting [88-90] as well as a low compliance which is discussed to be reinforced by the long treatment durations or the negative side effects [87, 91-97]. Substances that not only contribute to a healthy aging but also prevent and treat existing diseases are attracting growing interest.

7. Polyphenols and Nutraceuticals

By treating inflammatory disorders, more and more focus is put on natural substances, referred to as “nutraceutical”. “Nutrition” and “pharmaceutical” were thereby combined to the term, implicating a health supporting/maintaining property for these substances [98, 99]. Figure 4 gives an overview over several groups of secondary plant substances as well as food components with potential positive health impact that are currently discussed for usage as nutraceuticals in the prevention and treatment of disease related to MC, as they showed immunomodulatory effects *in vitro* and *in vivo* [100, 101].

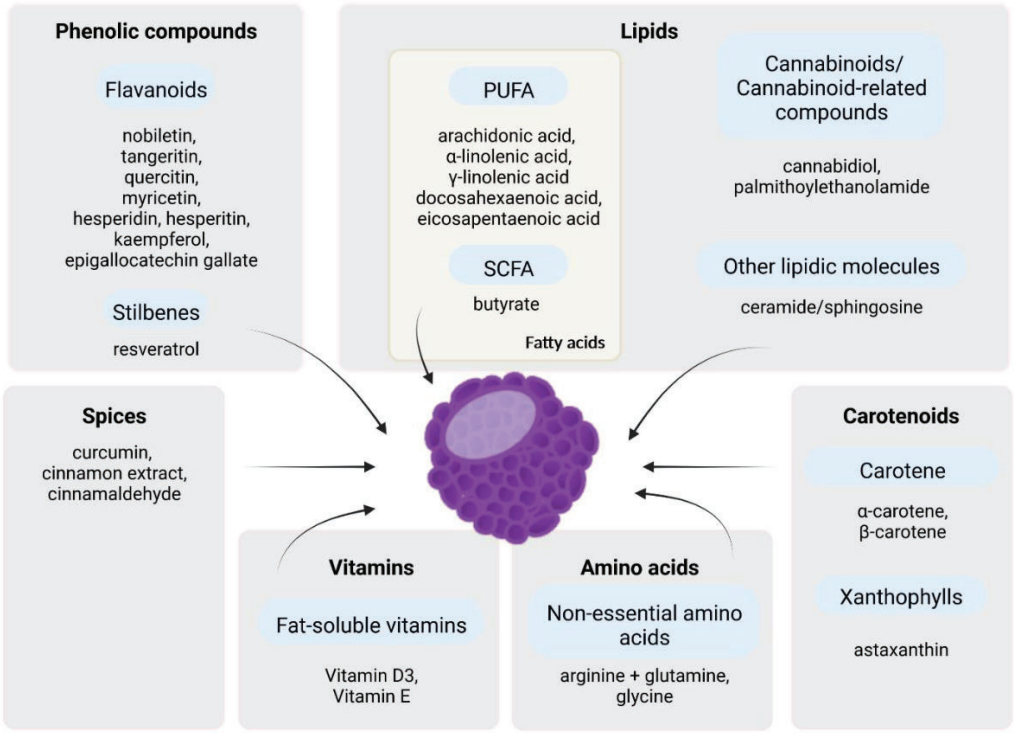

Figure 4 – Food components that were shown to have immunomodulatory effects on mast cells *in vitro* and *in vivo*. Different groups of secondary plant substances were shown to have immunomodulatory effects on different MC models. These are amongst others phenolic compounds, lipids, spices, vitamins, amino acids or carotenoids (adapted and modified from [100, 101]; created with BioRender.com).

Polyphenols were numerously shown to have anti-cancer effects [102] as well as anti-inflammatory [103], antioxidant [104] or neuroprotective [105] properties, as reviewed elsewhere. Therefore, these substances are getting more and more into focus in the nutricosmetic [106], nutritional [107] and medicine [108, 109] field for preventive aspects or treatment alternatives, but large and adequate human data from clinical trials are lacking.

One of the most studied polyphenols in terms of health-promoting effects is resveratrol (trans-3,5,4′-trihydroxystilbene) (RESV). This stilbene is found in > 70 plant species like nuts, berries and most abundantly in grapes [110].

The following questions were addressed to in this thesis:

- Do small natural components like resveratrol or nobiletin show anti-inflammatory effects *in vitro* on human intestinal mast cells (hiMC)?
- Do small natural components like resveratrol affect the signaling molecules ERK1/2 and STAT3 in the mitochondria of hiMC?
- Does resveratrol show anti-inflammatory effects on MC associated intestinal dieseases *in vivo* like ovalbumin (OVA)-induced enteritis or murine IL-$10^{-/-}$ colitis?
- What is the potential of resveratrol for use as a nutraceutical in MC mediated allergic reactions and what are the challenges coming along in using resveratrol as such?

RESULTS

1. Effects of small natural molecules on human intestinal mast cells and mitochondrial signaling molecules

It is well known that resveratrol shows a wide range of immunomodulatory effects *in vitro* [111-113], not only in MC models and MC associated diseases [22]. However, since MC are the main effector cells of allergies, we wanted to investigate the immunomodulatory effects of resveratrol on MC purified from human intestinal tissue [114]. Inhibitory effects on human intestinal mast cells (hiMC) have already been demonstrated for the flavonoids nobiletin and tangeritin [115].

MC degranulation, leading to the release of pre-stored or *de novo* synthesized mediators, requires mitochondrial translocation to the sites of exocytosis as well as ATP from OXPHOS in mitochondria [36]. Previous studies have shown that signaling molecules like STAT3 impact ATP production and therefore influencing MC degranulation [37, 38]. ERK1/2 is responsible for the activation of STAT3, as it phosphorylates mitochondrial STAT3 on its serine residue 727 [37]. Thus, we aimed to isolate mitochondrial fractions from hiMC and to further detect signaling molecules in these fractions.

The aim of our study was to clarify whether resveratrol shows immunomodulatory effects on hiMC, whether it is possible to isolate mitochondrial fractions from hiMC and whether activated STAT3 and ERK1/2 can be found in mitochondrial hiMC fractions. If so, we wanted to answer the question if small natural molecules like resveratrol and nobiletin influence the activation of these signaling molecules. We were able to isolate mitochondrial fractions from small numbers of hiMC and found that phosphorylation of ERK1/2 and STAT3 was inhibited by resveratrol in both mitochondrial and nuclear fractions of hiMC. Further, MC degranulation as well as cytokine and chemokine release were inhibited by treatment of the cells with resveratrol.

The article was published 2021 in “International Journal of Molecular Sciences” [116].

International Journal of
Molecular Sciences

MDPI

Article

Resveratrol Is a Natural Inhibitor of Human Intestinal Mast Cell Activation and Phosphorylation of Mitochondrial ERK1/2 and STAT3

Sabrina Bilotta [1], Lakshmi Bhargavi Paruchuru [2], Katharina Feilhauer [3], Jörg Köninger [3] and Axel Lorentz [1,*]

[1] Department of Nutritional Medicine, University of Hohenheim, Fruwirthstraße 12, 70593 Stuttgart, Germany; sabrina.bilotta@uni-hohenheim.de
[2] Department of Biochemistry and Molecular Biology, Institute for Medical Research—Israel-Canada, The Hebrew University of Jerusalem, Jerusalem 91120, Israel; bhargavi.lakshmi@mail.huji.ac.il
[3] Clinic for Visceral Surgery, Katharinenhospital, Kriegsbergstraße 60, 70174 Stuttgart, Germany; k.feilhauer@klinikum-stuttgart.de (K.F.); j.koeninger@klinikum-stuttgart.de (J.K.)
* Correspondence: lorentz@uni-hohenheim.de; Tel.: +49-711-459-24391

Abstract: Mast cells play a critical role as main effector cells in allergic and other inflammatory diseases. Usage of anti-inflammatory nutraceuticals could be of interest for affected patients. Resveratrol, a natural polyphenol found in red grapes, is known for its positive properties. Here, we analyzed the effects of resveratrol on FcεRI-mediated activation of mature human mast cells isolated from intestinal tissue (hiMC). Resveratrol inhibited degranulation and expression of cytokines and chemokines such as CXCL8, CCL2, CCL3, CCL4, and TNF-α in a dose-dependent manner. Further, resveratrol inhibited the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and signal transducer and activator of transcription (STAT) 3. ERK1/2 is known to be involved in cytokine expression of hiMC and to directly interact with STAT3. Mitochondrial STAT3 is phosphorylated by ERK1/2 and contributes to mast cell degranulation. We were able to isolate mitochondrial fractions from small hiMC numbers and could show that activation of mitochondrial STAT3 and ERK1/2 in hiMC was also inhibited by resveratrol. Our results indicate that resveratrol inhibits hiMC activation by inhibiting the phosphorylation of mitochondrial and nuclear ERK1/2 and STAT3, and it could be considered as an anti-inflammatory nutraceutical in the treatment of mast cell-associated diseases.

Keywords: mast cells; allergy; nutraceuticals; resveratrol; polyphenols; ERK1/2; STAT3; mitochondrial signaling

Citation: Bilotta, S.; Paruchuru, L.B.; Feilhauer, K.; Köninger, J.; Lorentz, A. Resveratrol Is a Natural Inhibitor of Human Intestinal Mast Cell Activation and Phosphorylation of Mitochondrial ERK1/2 and STAT3. *Int. J. Mol. Sci.* **2021**, *22*, 7640. https://doi.org/10.3390/ijms22147640

Academic Editor: Margarita Martín

Received: 15 June 2021
Accepted: 14 July 2021
Published: 16 July 2021

1. Introduction

Mast cells (MC) are key effector cells of type I allergic reactions; thus, they are closely related to allergic diseases, such as food allergies, as well as being linked to neuroimmune and inflammatory disorders, such as intestinal diseases [1–3]. Their main pro-inflammatory property is the release of inflammatory mediators such as pre-stored histamine or proteases, as well as de novo-synthesized cytokines or lipid mediators, after activation via diverse stimuli, of which the most important activation signal is the IgE-dependent stimulation of FcεRI IgE-receptor crosslinking by antigens [4]. The prevalence of allergies or intestinal diseases has increased in western countries in recent decades [5–7], thus simultaneously increasing patients' need for specific pharmaceutic medication that is often associated with negative side effects [8]. Natural substances could be a good alternative or additive therapy, and they are associated with better compliance. In the context of so-called *nutraceuticals*, the polyphenol resveratrol could be of special interest because of its beneficial immunomodulatory effects [9].

Resveratrol (trans-3,4′,5 trihydroxystilbene, trans-Resveratrol) is mainly found in grapes, berries, or peanuts. In context of allergies, the polyphenolic compound was one amongst others that was able to prevent the development of a food allergy in mice [10],

ameliorating the effects of atopic dermatitis and allergic rhinitis [11,12]. The anti-allergic and anti-inflammatory effects of resveratrol on different types of mast cell models have been shown previously [13–15]. Resveratrol suppressed IL-6 and TNF-α expression in mouse bone marrow-derived mast cells [13]. In the rat basophilic leukemia mast cell line (RBL-2H3), resveratrol was found to diminish β-hexosaminidase and histamine release [16]. Further, resveratrol was found to inhibit human eosinophil degranulation, as well as phosphorylation of protein kinases p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) [17]. In children and adults with allergic rhinitis, intranasal administered resveratrol ameliorated clinical symptoms [18,19].

Degranulation of mast cells requires mitochondrial translocation to the site of exocytosis [20], suggesting the involvement of mitochondrial oxidative phosphorylation (OXPHOS) in mast cell exocytosis. In parallel, mitochondrial signal transducer and activator of transcription (STAT) 3 was found to be involved in ATP production by influencing the electron transport chain [21]. Moreover, STAT3 was shown and to be essential for immunologically mediated degranulation of human and mouse mast cells, as well as RBL-2H3 cells [22]. In IgE-antigen-activated RBL-2H3 cells, mitochondrial STAT3 was found to be phosphorylated by ERK1/2 on serine residue S727 [22]. Furthermore, we and others found that citrus flavonoids, especially nobiletin, are inhibitors of ERK1/2 and STAT3 [23,24] that downregulate mast cell degranulation, suggesting that nonpeptidic small molecules, such as polyphenols, are able to inhibit mast cells by downregulation of mitochondrial activity and the inhibition of ERK1/2 or STAT3.

The important role of signaling molecules such as ERK1/2 and STAT3 makes them potential targets for alternative natural-based medication, referred to as *nutraceuticals*, in the treatment of diseases involving mast cells. Here, we examine the immunomodulatory role of resveratrol on human intestinal mast cells (hiMC), as well as on the involved signaling molecules. We show that resveratrol strongly inhibits mast cell activation and downregulates phosphorylated ERK1/2 and STAT3 in mitochondrial fractions of hiMC.

2. Results

2.1. Resveratrol Has No Toxic Effect on hiMC and Inhibits Mast Cell Degranulation and Chemokine Expression in a Dose-Dependent Manner

Mast cell activity is reported to be inconsistently affected by treatment with resveratrol [14,16,25,26] in several murine and human mast cell models. Thus, we aimed to investigate the effect of this polyphenol on primary mature human mast cells. We started to analyze whether resveratrol had any effect on cell viability of mast cells isolated from human intestinal tissue. HiMCs were incubated with 1–100 µM of resveratrol, concentrations previously used in mast cell models, for 24 h. Cell viability was determined by living cell counting and cytotoxicity was measured by absorbance of the MTT formazan product. Resveratrol did not show cytotoxic effects on cells when incubated for 24 h (Figure 1A, Supplemental Figure S1). To examine the effect of resveratrol on mediator release and gene expression, hiMCs were treated with 1–100 µM of resveratrol 1 h before stimulation via FcεRI crosslinking for 90 min. Degranulation, measured as β-hexosaminidase release, was found to be reduced by resveratrol in a dose-dependent manner. Thus, concentrations beginning at 10 µM significantly attenuated degranulation, showing the strongest effects at 100 µM (Figure 1B). To ascertain whether resveratrol also dose-dependently affected expression of de novo-synthesized mediators such as cytokines, we analyzed mRNA expression of the chemokine genes *Cxcl8*, *Ccl2*, *Ccl3*, and *Ccl4*. Treatment with concentrations of 5 µM of resveratrol or higher resulted in significant downregulation of *Cxcl8*, and *Ccl2*, *Ccl3*, and *Ccl4* were dose-dependently downregulated by concentrations of 25 µM and higher (Figure 1C–F).

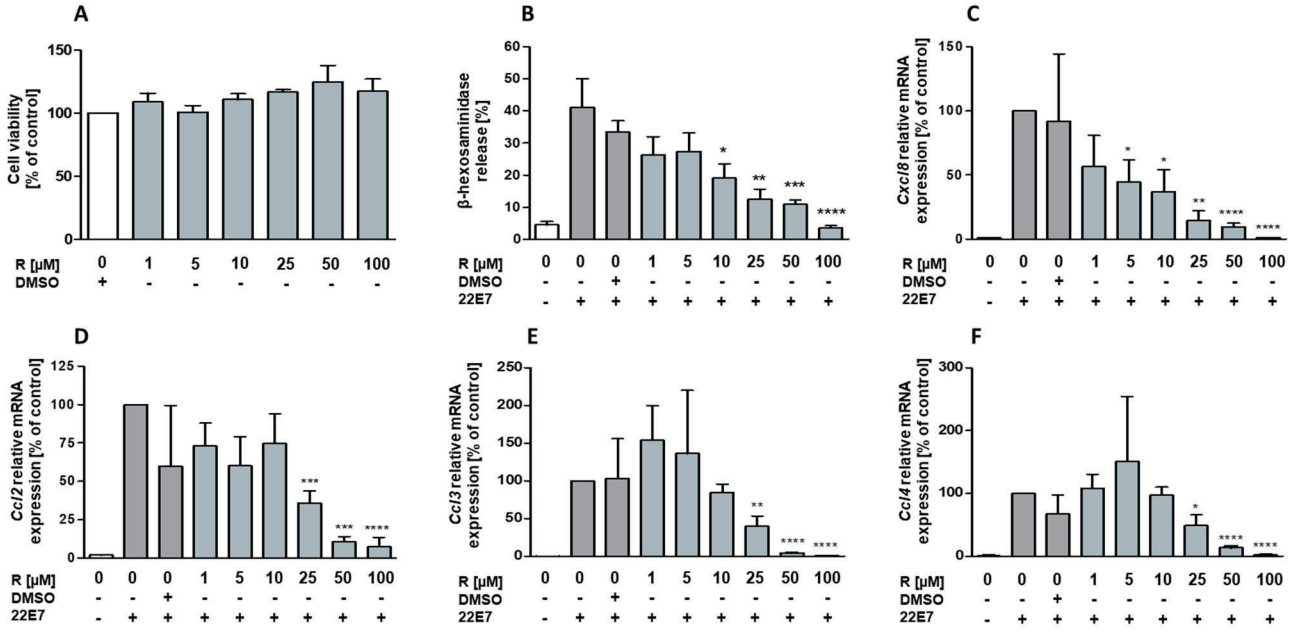

Figure 1. Cell viability, degranulation, and chemokine expression in human intestinal mast cells (hiMC) following treatment with resveratrol. Evaluation of cytotoxic effects of resveratrol on hiMC (**A**). Cells/well were incubated with 1, 5, 10, 25, 50, and 100 µM of resveratrol and the corresponding DMSO control for 24 h. After incubation, living cells in percent of the DMSO control is shown ($n = 3$). Release of β-hexosaminidase by hiMC (**B**) ($n = 6$) and mRNA expression of *Cxcl8* (**C**) ($n = 4$), *Ccl2* (**D**) ($n = 6$), *Ccl3* (**E**) ($n = 6$), and *Ccl4* (**F**) ($n = 6$). Cells were incubated with 1–100 µM of resveratrol for 60 min prior to stimulation by FcεRI crosslinking using 100 ng/mL of monoclonal antibody (mAb) 22E7 for 90 min. Controls were treated with the corresponding concentration of the vehicle DMSO. Values are mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to induced control treated with DMSO.

2.2. Resveratrol and Nobiletin Act Similarly to STAT3 Inhibitor on Mast Cell Activity

Resveratrol and the citrus flavonoid nobiletin were able to inhibit mast cell degranulation [23]. Moreover, STAT3 in mitochondria was found to be an important molecule required for this process by directly influencing ATP production [22]. To investigate the inhibition of mast cells by polyphenols, we examined the effects of nobiletin and resveratrol in comparison to STAT3 inhibitor stattic on mast cell degranulation and chemokine mRNA expression in hiMC. We found that the FcεRI-mediated degranulation of mast cells was totally inhibited by treatment with stattic and almost totally inhibited by treatment with resveratrol. Treatment with nobiletin significantly reduced mast cell degranulation but to a lesser extent (Figure 2A). Additionally, expression of *Cxcl8*, *Ccl2*, *Ccl3*, *Ccl4*, and of *Tnf-α* were determined in response to treatment with stattic, nobiletin, and resveratrol before stimulation of mast cells by FcεRI crosslinking. As shown in Figure 2B–F, stattic and resveratrol inhibited the expression of all cytokines by almost 100% compared to the stimulated control in hiMC. Again, the effect of nobiletin was less pronounced.

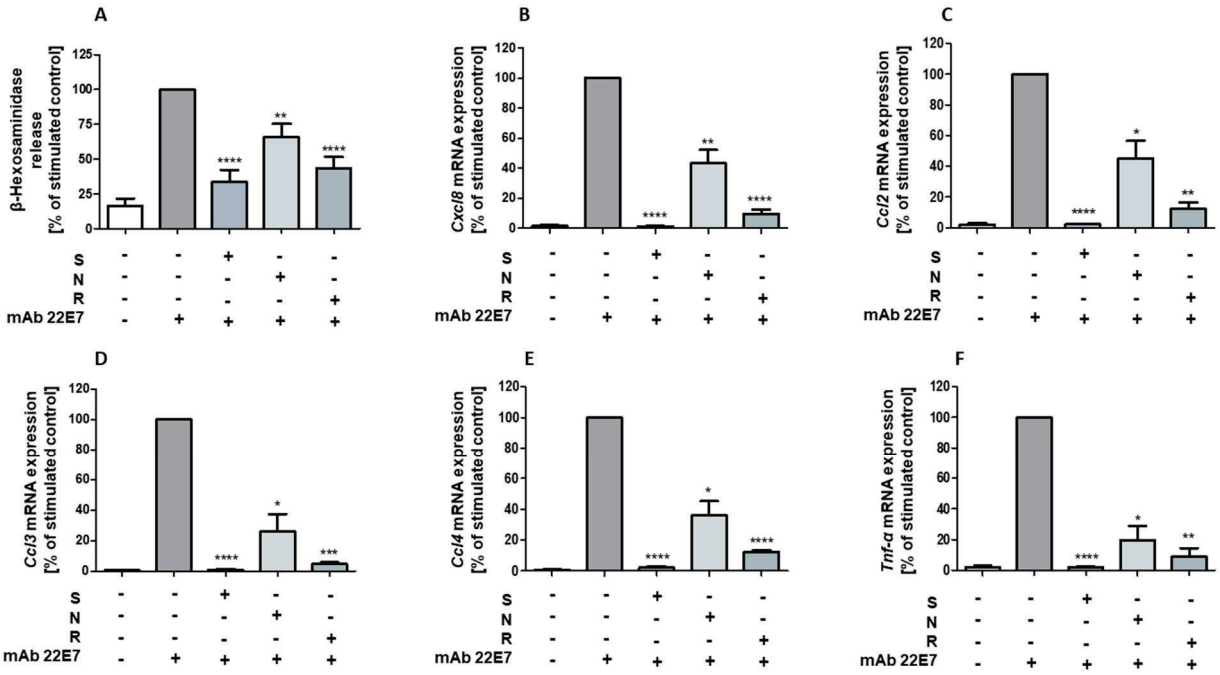

Figure 2. Degranulation and chemokine expression in hiMC following treatment with stattic, nobiletin, and resveratrol. Release of β-hexosaminidase (**A**) ($n = 9$) and mRNA expression of Cxcl8 (**B**) ($n = 4$), Ccl2 (**C**) ($n = 3$), Ccl3 (**D**) ($n = 3$), Ccl4 (**E**) ($n = 3$), and Tnf-α (**F**) ($n = 3$) by hiMC following treatment with 60 µM of stattic (S), 45 µM of nobiletin (N), or 50 µM of resveratrol (R), or corresponding concentrations of the vehicle DMSO (control). Cells were stimulated by FcεRI crosslinking using 100 ng/mL of mAb 22E7 for 5–90 min (**A**) and 90 min (**B**–**F**). Results are shown in % of stimulated control. Values are mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

2.3. Phosphorylation of STAT3 and ERK1/2 Is Diminished by Resveratrol and, to a Lesser Extent, by Nobiletin

Resveratrol and, to a lesser extent, nobiletin show inhibitory effects on degranulation and cytokine expression in hiMCs such as STAT3 inhibitor stattic. Thus, we next analyzed the effects of resveratrol and nobiletin on the phosphorylation of STAT3 in hiMC in response to FcεRI crosslinking. As expected, strong inhibition of phosphorylated STAT3 was achieved by STAT3 inhibitor stattic. Importantly, resveratrol also inhibited phosphorylation of STAT3. Treatment with nobiletin resulted in reduced phosphorylation, which was not significant (Figure 3A). It is already known that mitochondrial STAT3 is phosphorylated by ERK1/2 [22] and that ERK1/2 translocate to mitochondria, with an impact on the regulation of mitochondrial activity. Therefore, we examined the effects of resveratrol, nobiletin, and stattic on phosphorylation of ERK1/2 in hiMC following FcεRI crosslinking. Treatment with resveratrol results in almost complete inhibition of ERK1/2 activation. Additionally, inhibition by stattic and nobiletin was significant but not as strong as it was with resveratrol (Figure 3B).

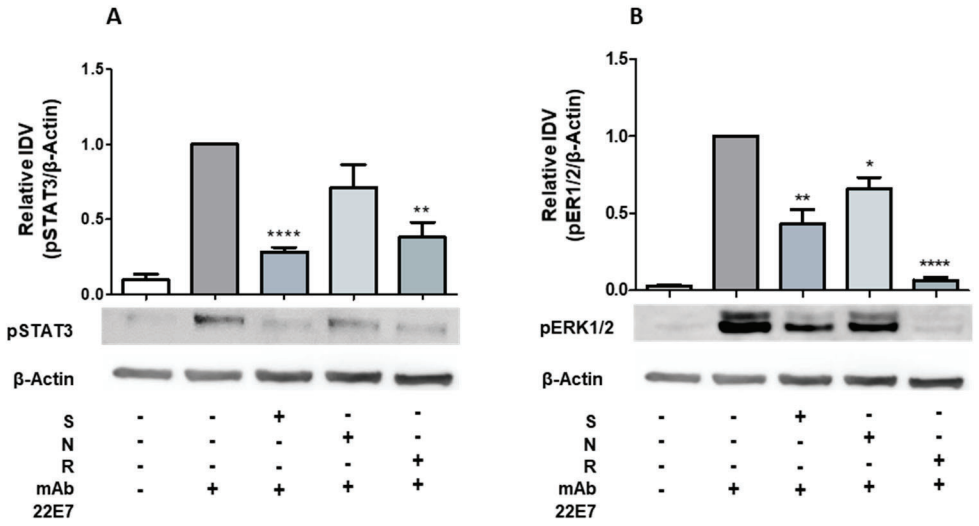

Figure 3. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase 1/2 (ERK1/2) in hiMC following treatment with stattic, nobiletin, and resveratrol. Western blot analyses of (**A**) phosphorylated STAT3 ($n = 5$) and (**B**) phosphorylated ERK1/2 ($n = 4$) in hiMC. HiMCs were treated with 60 μM of stattic (S), 45 μM of nobiletin (N), 50 μM of resveratrol (R), or corresponding concentrations of the vehicle DMSO (control) before stimulation by FcεRI crosslinking using 100 ng/mL of mAb 22E7 for 5 min. Representative pictures and densitometric analyses in relation to stimulated control are shown. Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

2.4. Phosphorylated STAT3 and ERK1/2 Are Detectable in Mitochondrial Fractions of hiMC after FcεRI Crosslinking and Are Inhibited by Resveratrol

Mast cell degranulation and mitochondrial activity were shown to be closely related [20]. Other than its canonical role, STAT3 is phosphorylated by ERK1/2 in mitochondrial fractions of RBL-2H3 cells [22], and it directly contributes to exocytosis. To prove that polyphenols directly affect phosphorylated STAT3 and ERK1/2 in mitochondria, these cell organelles had to be isolated from mast cells by subcellular fractionation. First, fractionation had to be optimized by reducing cell numbers because very high cell numbers, as suggested by available protocols, are not reachable using mature human mast cells from intestinal tissue [22,27,28]. PDH E1α was used as mitochondrial marker and HDAC-1 was used to show the nuclear fraction. It should be noted that the nuclear fraction also contains crude cell extract, which is not separated during fractionation. Having optimized the subcellular fractionation protocol for mitochondrial fractions down to 2×10^6 hiMC per condition, we attempted to detect phosphorylated STAT3 and phosphorylated ERK1/2 in mitochondrial fractions. We were able to detect phosphorylated ERK1/2 (Figure 4A) and phosphorylated STAT3 (Figure 4B) in mitochondrial fractions of hiMC. More importantly, we found that treatment of hiMC with resveratrol before stimulation by FcεRI crosslinking results in the inhibition of activated ERK1/2 and STAT3 phosphorylation in mitochondrial fractions of hiMC.

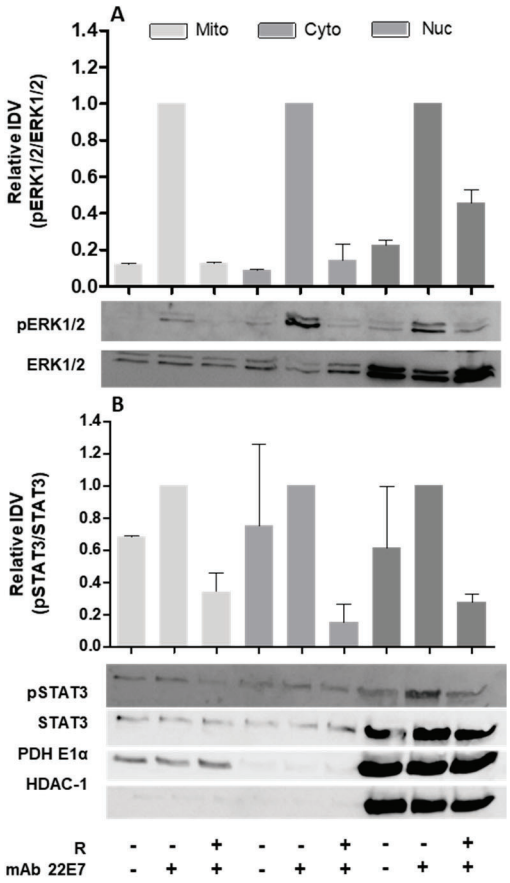

Figure 4. Phosphorylation of STAT3 and ERK1/2 in nuclear and mitochondrial fractions of hiMC following treatment with resveratrol. Western blot analyses of two independent experiments of (**A**) phosphorylated ERK1/2 and (**B**) phosphorylated STAT3 after subcellular fractionation in pure mitochondria (Mito), cytosol fraction (Cyto), and crude nuclear fraction (Nuc), respectively. HiMCs were treated with 50 μM of resveratrol (R), or corresponding concentrations of the vehicle DMSO (control), and stimulated by FcεRI crosslinking with 100 ng/mL of mAb 22E7 for 5 min. Exemplary pictures and densitometric analyses in relation to stimulated control for Western blots of phosphorylated ERK1/2 and total ERK1/2, and phosphorylated STAT3 and total STAT3, are shown. Values are mean ± SEM (n = 2).

3. Discussion

In the present study, we show that resveratrol is a potent inhibitor of mature human mast cell activation. Resveratrol shows strong inhibitory effects on the release of pre-stored mediators and the expression of de novo-synthesized mediators. Further, we could show that resveratrol inhibits activation of ERK1/2 and STAT3 in both nuclear and mitochondrial fractions of hiMC. Resveratrol shows stronger inhibitory effects, e.g., compared to nobiletin, a polymethoxyflavone from citrus peel. Thus, resveratrol might be a potential natural medication alternative, referred to as *nutraceutical*, in the treatment of mast cell-associated diseases, such as allergies.

The effect of polyphenols such as resveratrol in relation to IgE-dependent MC activation has been analyzed in different mast cell models. As such, resveratrol was found to potently inhibit mast cell degranulation in RBL-2H3 cells (up to 50%) [16,25] and mouse BMMC (50%) [26]. In primary human skin mast cells, degranulation was not affected by low concentrations of resveratrol (<50 μM), but only in the range of 50–100 μM. We found inhibition of degranulation in hiMC starting at 10 μM, with the strongest effect up to almost 100% at 100 μM of resveratrol. In contrast to human skin mast cells, we did not detect enhanced expression of TNF-α in hiMC following treatment with low concentrations of resveratrol (<10 μM) (not shown) [15]. Resveratrol strongly decreased expression of

Cxcl8, *Ccl2*, *Ccl3*, *Ccl4*, and *Tnf-α* in hiMC. In human mast cell line HMC-1, picetannol, a resveratrol metabolite, was also able to reduce the gene expression of *Tnf-α* and *Cxcl8* [29].

Several chemokines are increased in inflammatory processes such as allergies [30]. CCL2, CCL3, and CCL4 are regulatory factors in immune and endothelial regulation, as well as in chemotaxis [31]. It was previously shown that CCL2 recruits macrophages to sites of inflammation after allergen exposure [32], and that the blocking of CCL2 signaling pathway prevents Th2 inflammatory response in allergic asthma [33]. TNF-α and CXCL8 were reported to serve as important inflammatory cytokines by attracting neutrophils and basophils and promoting inflammatory reactions, not only in relation to allergies [34–36].

The induction of CCL2 and CXCL8 is regulated by the MAPK signaling pathway. It was found that the application of ERK-specific inhibitors on human eosinophils reduced the release of CCL2 and CXCL8 [37]. In the human MC line LAD2, and in human cord blood-derived MC, expression and release of CCL2 and CCL5 was induced by IL-33; this induction was due to the activation of the MAPK signaling pathway, even though ERK showed no direct influence on chemokine expression for these cells [38]. In LAD2 cells, CCL2 production was induced by C3a complement component-dependent MC activation, which was shown to be inhibited by usage of the U0126 inhibitor of MEK-induced ERK phosphorylation [39]. In contrast, in IgE-activated RBL-2H3 cells, CCL2 production was not affected by ERK1/2 inhibition [40]. In hiMC, the MAPK family is well known to be involved in cytokine expression [23,41–43]. In addition, we found that flavonoids nobiletin and, to a lesser extent, tangeritin show inhibitory effects on ERK1/2 phosphorylation, as well as on *Cxcl8*, *Ccl3*, and *Ccl4* expression [23]. Here, we show that resveratrol inhibits FcεRI-mediated phosphorylation of ERK1/2 and the expressions of *Cxcl8*, *Ccl2*, *Ccl3*, and *Ccl4* in hiMC. Inhibition of ERK1/2 phosphorylation by resveratrol was also found in HMC-1 cells and at a high concentration in human skin mast cells [14,15]. However, it should be noted that the inhibitory effect of resveratrol on ERK1/2 was very pronounced, but not limited to it. Phosphorylation of other IgE-dependently activated kinases, such as Akt or JNK in hiMC (Supplemental Figure S2), or Akt and p38 in human skin mast cells, was also reduced in response to treatment with resveratrol [15].

MAP kinase ERK1/2 is further known to directly affect STAT3 [22], which in turn is involved in ATP production in mitochondria. It is known that MC degranulation requires mitochondrial translocation to sites of exocytosis and that OXPHOS may be a central process in MC activation [20]. We therefore tested whether resveratrol affects phosphorylation of ERK1/2 and STAT3. Indeed, resveratrol displays inhibitory effects on both molecules. Aside from its canonical role as a transcription factor, STAT3 was shown to participate in electron transport in the process of OXPHOS-dependent ATP production in mitochondria [21]. ATP serves as an energy source for MC degranulation. Two STAT3 inhibitors, mitocur-1 and -3 based on curcumin, were able to affect degranulation and cytokine release in murine and primary human mast cells, and diminished ATP levels in cells cultured in glucose-free medium, indicating a direct effect on mitochondrial ATP production. Additionally, both curcumin-based inhibitors decreased histamine release in acute anaphylaxis in mice [44]. Curcumin is a polyphenol obtained from turmeric and is intensively discussed as alternative medication due to its positive biological properties [45]. STAT3 inhibitors mitocur-1 and mitocur-3 are directed against mitochondrial STAT3, reducing its phosphorylation on serine 727 residue. A resveratrol–caffeic acid hybrid was detected to affect and inhibit acetylation, as well as the phosphorylation of STAT3 on tyrosine residue T705, in two human cancer cell lines [46]. In hiMC, resveratrol was able to inhibit the activation of STAT3-S727 in both nuclear and mitochondrial fractions.

Isolation of hiMC from surgical tissue does not provide large cell numbers. Thus, to analyze mitochondrial fractions from mature hiMC, we had to optimize the fractionation for comparatively low cell numbers. Fortunately, we were able to isolate mitochondrial fractions from small hiMC numbers and could show that phosphorylated ERK1/2 and STAT3 were present in this fraction. Resveratrol was found to inhibit the phosphorylation of ERK1/2 and STAT3 in mitochondria of hiMC activated via crosslinking of the FcεRI

receptor. Occurrence and activation of ERK1/2 and STAT3 in mitochondria suggest the importance of signaling molecules present in mitochondria in terms of MC activity.

The increase in MC-associated diseases requires novel treatment possibilities. Notably, negative side effects related to conventional medication may be overcome with natural-based alternatives, thus increasing patients' acceptance. We have previously shown that plant-derived substances have the potential to inhibit the release of MC-specific mediators in hiMC. Cinnamon extract could reduce degranulation down to 20% in hiMC after IgE-dependent activation, as well as completely inhibit the expression of *Cxcl8*, *Ccl2*, *Ccl3*, *Ccl4*, and *Tnf-α* [23,42]. Cinnamaldehyde was thereby shown to be the active compound of cinnamon extract, leading to its anti-inflammatory actions in hiMC [47]. However, citrus flavonoids, such as nobiletin and tangeritin or stilbene resveratrol, may be more acceptable to patients than cinnamon extract or cinnamaldehyde. We found that resveratrol shows stronger inhibitory effects on hiMC than citrus flavonoids. *Citrus tachibana* leaf extract, with its components nobiletin and tangeritin, improved OVA-induced allergic symptoms such as diarrhea and rectal temperature [48]. Application of resveratrol for 13 days improved the same parameters in OVA-treated mice and reduced histamine and MC protease 1 in serum [16]. These observations show that inflammatory disorders associated with MC can be alleviated with natural occurring plant substances, and that resveratrol can be a highly potent anti-allergic plant substance.

In summary, our results show a strong inhibitory effect of resveratrol on hiMC degranulation and chemokine expression. These effects seem to be mediated by inhibition of ERK1/2 and STAT3. The data suggest that resveratrol could be considered as a potential natural-based anti-allergic component, a *nutraceutical*, for the treatment of MC-associated disorders such as allergies.

4. Materials and Methods

4.1. Isolation and Culture of hiMC

HiMCs were isolated from surgical tissue from patients who underwent bowel resection, as previously described [49]. Permission to conduct the study was obtained by the local ethical committee. Tissue underwent mechanical shredding and enzymatic digestion with pronase (Serva, Heidelberg, Germany) and collagenase D (Nordmark Biochemicals, Uetersen, Germany). After overnight culture of obtained cell suspension in RPMI 1640+GlutaMax^{TM} (Gibco Invitrogen, Paisley, OR, USA) with 10% FBS (Merck, Darmstadt, Germany), 100 µg/mL streptomycin, 100 U/mL penicillin (HyClone^{TM} Laboratories, South Logan, Utah, USA), 100 µg/mL gentamycin, and 2.5 µg/mL amphotericin B (CarlRoth Karlsruhe, Germany), enrichment of cells by magnetic cell separation of c-Kit+ (CD117) cells was conducted using *CD117 microbead kit* after *dead cell removal kit* (MACS^{TM} system, Miltenyi Biotech, Bergisch Gladbach, Germany). Pure hiMCs were cultured with 25 ng/mL stem cell factor (SCF) (PeproTech, Hamburg, Germany) and 2 ng/mL IL-4 (PeproTech) for at least 14 days before use in experiments.

4.2. Cell Viability

Next, 5 × 10^4 hiMCs per well were incubated in a 48-well plate in the presence of 1, 5, 10, 25, 50, or 100 µM resveratrol (Merck, Darmstadt, Germany), respectively, or the vehicle DMSO (CarlRoth). After 24 h, living cells were counted after trypan blue staining. Additionally, an MTT assay was performed. For that, 25 µL of MTT solvent (Merck) was added to each well and incubated for 3 h. Supernatant was discarded, 100 µL of lysis solution was added to each well and gently mixed, and absorbance of MTT formazan product was measured to detect the amount of substrate converted by living cells.

4.3. Treatment of hiMC

Cells were treated with 1, 5, 10, 25, 50, or 100 µM resveratrol or 45 µM nobiletin (Indofine Chemical, Hillsborough, NJ, USA) 1 h prior to stimulation by FcεRI crosslinking using 100 ng/mL monoclonal antibody (mAb) 22E7 directed against the FcεRI α-chain

(Genentech, South Francisco, CA, USA). Cells were stimulated for 5 or 90 min to analyze degranulation, for 90 min to analyze mRNA expression, and for 5 min to detect activated signaling molecules. To analyze STAT3 activation, hiMCs were pre-incubated for 20 min with 60 µM STAT3 inhibitor stattic (Merck) prior to stimulation by FcεRI crosslinking. Unstimulated controls contained the same concentrations of vehicle DMSO.

4.4. Degranulation

Degranulation of MC was measured by determining the amount of released β-hexosaminidase in supernatants by a color enzyme assay [50].

4.5. RNA Preparation and Real-Time RT-PCR

Total RNA was obtained by using an EXTRACTME TOTAL RNA kit (blirt, Gdansk, Poland). Real-time RT-PCR reactions were performed in optical tubes containing 1 µL of cDNA template, 0.125 µL each sense and anti-sense primer, 4 µL of H_2O, and 5 µL of SsoFast™ EVAGreen Supermix (Bio-Rad Laboratories, Feldkirchen, Germany). Reaction mixture without cDNA was used as negative control. Relative quantification ($2^{-\Delta\Delta Ct}$) was performed using glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) housekeeping gene as reference. Sense and antisense primer sequences were: *Gapdh*: 5′-TGG TCT CCT CTG ACT TCA AC-3′, 5′-CCT GTT GCT GTA GCC AAA TT-3′, product size: 128 bp; *Cxcl8*: 5′-CTG AGA GTG ATT GAG AGT GG-3′, 5′-ACA ACC CTC TGC ACC CAG TT-3′, product size: 113 bp; *Ccl2*: 5′-CTT CTG TGC CTG CTG CTC AT-3′, 5′-CGG AGT TTG GGT TTG CTT GTC-3′, product size: 273 bp; *Ccl3*: 5′-CTC TGC ATC ACT TGC TGC TGA CAC-3′, 5′-CAC TCA GCT CCA GGT CGC TGA C-3′, product size: 212 bp; *Ccl4*: 5′- GCT AGT AGC TGC CTT CTG CTC TCC-3′, 5′-CAG TTC CAG CTG ATA CAC GTA CTC C-3′, product size: 238 bp; *Tnf-α*: 5′-CAG ATA GAT GGG CTC ATA CCA GGG-3′, 5′-GCC CTC TGG CCC AGG CAG TCA G-3′, product size: 377 bp (all Eurofins, Ebersberg, Germany). CFX 2.1 software and a CFX Connect Real-Time PCR System of Bio-Rad Laboratories were used.

4.6. Isolation of Mitochondria from hiMC

Subcellular fractionation protocol for the purification of hiMC mitochondrial fractions was adapted from Sharkia et al. [51] and modified for isolation working with low cell numbers of 2–5 × 10^6. Cell compartments were fractionated into mitochondria, nucleus, and cytosol by several ascending centrifugation and mechanical cell lysis using a syringe needle before lysis with RIPA buffer (0.01 mol/L Tris-Hcl, 1% deoxycholate, 0.1% SDS, 0.15 mol/L sodium chloride, 0.25 µmol/L phenylmethylsulfonylfluoride (all CarlRoth), and 1% Triton-X 100 (Merck)) containing protease inhibitor cocktail cOmplete™ Ultra Tablets Mini and phosphatase inhibitors phosSTOP™ (both Roche Diagnostics, Mannheim, Germany) and subsequent sonication. In brief, cell suspension was homogenized in buffer A (250 mM sucrose, 20 mM HEPES, 10 mM potassium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 1 mM EGTA (all CarlRoth), and 1 mM DTT (Invitrogen, Karlsruhe, Germany)) and passed through a syringe needle 10 times before centrifugation at 720× *g* for 5 min and 2000× *g* for 3 min. The obtained pellet was again homogenized with buffer A, pulled through a syringe needle 10 times, and centrifuged at 2000× *g* for 10 min before pellet lysis with RIPA buffer and sonication to obtain the nuclear fraction. Obtained supernatant from the first step was transferred to a clean tube and further centrifuged at 12,000× *g* for 10 min; obtained supernatant was marked as cytosol fraction and pellet homogenized with buffer A, and was pulled through a syringe 10 times before being centrifuged at 12,000× *g* for 10 min again. Mitochondrial fraction was lysed in RIPA buffer and by final sonication. All steps were performed at 4 °C.

4.7. Western Blot Analysis

Whole cell lysates were obtained by lysis of cells with extraction buffer containing 25 mM Tris pH7.4, 0.5 mM EDTA, 10 mM β-Mercaptoethanol (CarlRoth), and 0.05% Triton-X (Merck) supplemented with protease inhibitor cocktail cOmplete™ Ultra Tablets Mini

and phosphatase inhibitors phosSTOP™ (both Roche Diagnostics). Whole cell lysates or subcellular fractions were separated in a 12% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane (Immobilon®-P, CarlRoth) in 38.6 mmol/L glycine, 47.9 mmol/L tris base, 1.28 mmol/L SDS, and 20% methanol (CarlRoth, respectively) by blotting with Trans-Blot Cell (Bio-Rad). Membranes were blocked with 5% FBS in tris-buffered saline containing 0.1% Tween-20 (TBS-T) (CarlRoth) for at least 30 min at room temperature. Afterwards, membranes were probed with respective antibodies for phospho-STAT3 (S727), STAT3 (124H6), phospho-ERK1/2 (P44/42 MAPK, 137F5), HDAC-1 (D5C6U), phospho-Akt (4060s), phospho-SAP/JNK (2821) (Cell Signaling Technology®, Frankfurt, Germany), ERK1/2 (12D4) (Enzo®Life Sciences, Lausen, Switzerland), PDH-E1α (proteintech, St. Leon-Rot, Germany), or β-Actin (13E5) rabbit mAb (Cell Signaling Technology®) overnight at 4 °C, and the next day with respective HRP-linked secondary antibodies anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology®) for 60 min at room temperature. Visualization was performed by using SuperSignal™ West Duration Substrate (ThermoFisher Scientific, Bonn, Germany) and an electro-chemiluminescence detection system (FluorChem; Biozym Scientific, Hessisch Oldendorf, Germany). Signals were measured by a bioimaging analyzer (Alpha Innotech Corporation, San Leandro, CA, USA) and normalization was performed with β-Actin or the corresponding unphosphorylated signal molecule. For detection of several proteins, membranes were stripped in 25 mM glycin and 1% SDS in a water bath at 37 °C, and probed again with the respective antibody.

4.8. Statistics

Data are expressed as mean ± standard error of the mean (SEM). Student's *t*-test was used to analyze differences between two groups. GraphPad Prism scientific software version 5.0 (San Diego, CA, USA) was used for statistical analysis. Values of $p < 0.05$ were considered to be statistically significant.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22147640/s1.

Author Contributions: Conceptualization, A.L.; Resources, K.F. and J.K.; Methodology: S.B. and L.B.P.; Investigation, S.B.; Data Analysis, S.B. and A.L.; Supervision, A.L.; Writing—Original Draft preparation, S.B.; Writing—Review and Editing, A.L.; Project Administration, A.L.; Funding Acquisition, A.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by German-Israeli Foundation for Scientific Research and Development (GIF) (Grant No: I-1469-414.13/2018).

Institutional Review Board Statement: Surgery tissue specimen from patients undergoing bowel resection served for isolation of mature human mast cells. This study has been approved by the local ethics committee Stuttgart (F-2018-071; 23 October 2018) and has therefore been performed in accordance with the ethical standards. All subjects gave their informed consent before their inclusion.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Acknowledgments: The authors thank Yvonne Soltow for excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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2. Effects of resveratrol on mast cell mediated diseases

Increasing prevalence of gastrointestinal disorders like food allergies [49] or IBD [71, 73, 74], which are both closely related to MC activity [11, 117-121], leads to inevitability of new medication possibilities as conventional pharmaceutic medication may be associated with negative side effects like vomiting or nausea [88-90]. Even though the amount of medication available is a good way for treating these disorders, as it is well established and safe [122], application may be associated with low compliance [87, 91-97]. In this regard, natural substances like stilbenes or flavonoids showed promising effects on a wide range of MC-associated diseases which makes them a good alternative or additive treatment option [123-128]. Since the polyphenol resveratrol shows a high range of immunomodulatory effects [110], it could be of special interest in nutraceutical research.

Resveratrol was applied via drinking water for 28 days in the OVA setting or 90 days in the IL-$10^{-/-}$ setting. During the experimental phase we examined both effects of resveratrol on clinical symptoms and effects related on immunological changes in selected tissue sections after completion of the experiments.

The experiments were aimed to clarify whether resveratrol showed immunomodulatory effects on two different models of gastrointestinal disorders closely related to MC, namely OVA-induced allergic enteritis in BALB/c mice and murine IL-$10^{-/-}$ colitis. We found that oral application of resveratrol inhibited the increase of MC numbers in the colon and duodenum in both experimental settings. Effects of resveratrol administration were observed in the colon with regard to epithelial damage and in IL-$10^{-/-}$ colitis also with regard to cell infiltration as well as reduction of goblet cell numbers, which overall decreased to the level of the respective controls. Allergic enteritis resulted in a weaker symptomatology, and IL-$10^{-/-}$ animals showed a delayed appearance of the typical symptoms.

The article was published 2022 in “International Journal of Molecular Sciences” [129].

International Journal of
Molecular Sciences

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Resveratrol Treatment Prevents Increase of Mast Cells in Both Murine OVA Enteritis and IL-10$^{-/-}$ Colitis

Sabrina Bilotta, Julian Arbogast, Nadine Schart, Maurice Frei and Axel Lorentz *

Institute of Nutritional Medicine, University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany; sabrina.bilotta@uni-hohenheim.de (S.B.); julian.arbogast@uni-hohenheim.de (J.A.); nadine.schart@uni-hohenheim.de (N.S.); maurice.frei@uni-hohenheim.de (M.F.)
* Correspondence: lorentz@uni-hohenheim.de; Tel.: +49-711-459-24391

Abstract: Mast cells are involved in allergic and other inflammatory diseases. The polyphenol resveratrol is known for its anti-inflammatory properties and may be used as nutraceutical in mast cell associated diseases. We analyzed the effect of resveratrol on mast cells in vivo in ovalbumin-induced allergic enteritis as well as experimental colitis in IL-10$^{-/-}$ mice which received resveratrol via drinking water. Treatment with resveratrol prevented the increase in mast cells in both allergic enteritis and chronic colitis in duodenum as well as in colon. Further, it delayed the onset of diseases symptoms and ameliorated diseases associated parameters such as tissue damage as well as inflammatory cell infiltration in affected colon sections. In addition to the findings in vivo, resveratrol inhibited IgE-dependent degranulation and expression of pro-inflammatory cytokines such as TNF-α in IgE/DNP-activated as well as in LPS-activated bone marrow-derived mast cells. These results indicate that resveratrol may be considered as an anti-allergic and anti-inflammatory plant-derived component for the prevention or treatment of mast cell-associated disorders of the gastrointestinal tract.

Keywords: mast cells; food allergy; enteritis; IBD; colitis; resveratrol; polyphenols; nutraceuticals

Citation: Bilotta, S.; Arbogast, J.; Schart, N.; Frei, M.; Lorentz, A. Resveratrol Treatment Prevents Increase of Mast Cells in Both Murine OVA Enteritis and IL-10$^{-/-}$ Colitis. *Int. J. Mol. Sci.* **2022**, *23*, 1213. https://doi.org/10.3390/ijms23031213

Academic Editors: Sonia A.O. Santos, Armando J. D. Silvestre, Raphael Grougnet and Vessela Balabanova

Received: 22 December 2021
Accepted: 19 January 2022
Published: 21 January 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

The prevalence of gastrointestinal disorders such as food allergies or inflammatory bowel disease (IBD) has increased in western countries throughout the past decades [1,2]. Pharmaceutic medication can be associated with negative side effects such as vomiting or nausea for patients affected [3–5]. Although conventional drug therapy is well established and safe [6], generally low compliance has been reported [7–13]. Reasons for low compliance may be high costs, risk of adverse effects or long treatment durations [14]. Natural medication may be associated with none or less adverse effects [4]. Plant substances such as stilbenes, flavonoids and others may be a good alternative and additive therapy option to treat the clinical symptoms of allergy [15,16]. In the context of nutraceuticals, polyphenols such as resveratrol could be of special interest in the near future due to their wide beneficial immunomodulatory effects [17].

Mast cells (MC) are key effector cells of type I allergy and release pro-inflammatory mediators such as pre-stored histamine or de novo synthesized cytokines and lipid mediators in response to IgE-dependent stimulation via the FcεRI receptor crosslinking by antigens [18]. The role of MC and their mediators was numerously shown not only in vitro in different MC models [19–21] or in vivo in several allergic conditions [22–24] but also in humans [25–27]. Consequently, MC play an important role in gastrointestinal disorders due to food allergens [28]. Additionally, MC were shown to be involved in IBD [29–32] of which ulcerative colitis and Crohn's disease represent the most common disorders [32]. For example, the MC proteases MCPT-6 and Prss31 were found to be involved in the

formation of acute colitis [33–35] and MC numbers were found to be elevated in IBD patients and mice with colitis [36,37].

Resveratrol (trans-3,4′,5 trihydroxystilbene, trans-Resveratrol) is mainly found in grapes, berries or peanuts [38,39]. Numerous studies already showed the anti-inflammatory effects of resveratrol in the context of allergy and MC reactivity [24,40–44]. We have recently shown that resveratrol is a potent inhibitor of primary human intestinal mast cells' (hiMC) activity isolated from surgical tissue from patients undergoing bowel resection [40]. The anti-inflammatory effect of resveratrol was not only shown in vitro [24,40] but also in vivo in murine models of airway inflammation, atopic dermatitis or allergic rhinitis [41–44]. This was confirmed in humans by intranasal administration of resveratrol in children and adults suffering from allergic rhinitis [25,26].

Resveratrol treatment was found to attenuate clinical symptoms of ovalbumin (OVA)-induced allergic rhinitis in mice accompanied by a reduction in the release of inflammatory mediators [45]. Further, resveratrol treatment resulted in reduced cytokine concentrations in bronchoalveolar fluid (BALF) in mice with OVA-induced asthma [46]. Moreover, anti-allergic property of resveratrol was shown in OVA-induced model of food allergy by preventing onset of allergic symptoms as well as reduced IgE serum production [47]. Food allergy affects the gastrointestinal tract including local inflammations. These inflammations are amongst others associated with infiltration of immune cells such as MC into affected tissues [28,48,49]. In addition, intestinal inflammation such as IBD was also recognized to be correlated with MC numbers and activity [30,31,50]. We observed an increased number of MC and higher expression of MC proteases in IL-10$^{-/-}$ mice which develop a spontaneous form of chronic colitis due to the missing anti-inflammatory cytokine IL-10 [51–53]. Thus, we examined the immunomodulatory role of resveratrol on MC in both an OVA-induced allergic enteritis model and the IL-10$^{-/-}$ mouse.

2. Results

2.1. Resveratrol Inhibits Increase in MC in OVA-Enteritis

To induce allergic enteritis, animals were intraperitoneally (i.p.) sensitized twice with 50 μg and challenged six times with 50 mg OVA orally. Allergic disorders in the gut due to Th2 immune responses are known to be accompanied with diarrhea [28]. None of the animals receiving OVA showed signs of severe diarrhea (score 3), but overall stool score was significantly elevated in comparison to controls. First signs of stool softening appeared after gavage number 4 on experimental day 22. Overall, stool scores for OVA groups were significantly elevated in comparison to control animals (Figure 1A). In OVA-challenged mice, stool scores were the highest for OVA group receiving no additive. Ethanol and resveratrol receiving OVA groups thereby showed less high stool scores than those without additive and these observations were not different from effects observed in control groups (Figure 1A). Two animals of the OVA group receiving water died of an anaphylactic shock, after fifth and sixth OVA application, respectively, whereas all animals treated with resveratrol or the vehicle ethanol survived (Figure 1B).

Food allergy and enteritis are accompanied by histological changes and injury in the gut [50,54–57]. We found that OVA-induced allergic enteritis resembles a mild form of inflammation. All determined scores were relatively low (Figure 1C). Although resveratrol administration seemed to attenuate tissue damage, the results were not significant (Figure 1C). Nonetheless, in OVA-treated animals receiving the additive resveratrol, levels were diminished down to the levels of the control mice.

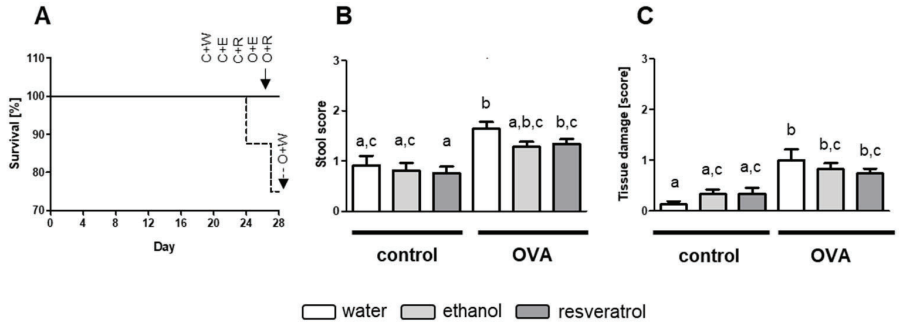

Figure 1. (**A**) Survival rate (%) of mice after 28 days, (**B**) stool score after first signs of diarrhea after 4th gavage of ovalbumin (OVA) and (**C**) scores for tissue damage in colon. Mice received water (white, (W)), 50 mg/kg bodyweight (BW) ethanol (light grey, (E)) or 50 mg/kg BW resveratrol (dark grey, (R)) via drinking water for 28 days and were intraperitoneally (i.p.) sensitized with 50 µg OVA in alum (1:2) on day 5 and 11 and further treated with or without (control, (**C**)) 50 mg OVA orally on day 15, 18, 20, 22, 24 and 27. Scores were determined in at least three hematoxylin and eosin (H&E) stained tissue sections, group size was n = 8, respectively. Values are mean ± SEM. Common letters indicate no significant difference between groups, different letters indicate significant change with at least $p < 0.05$. *p*-values for the respective data sets are shown in Figure S1.

The involvement of MC in enteritis induced by oral allergens was reported earlier [28]. Here, we found a significant increase in MC numbers in duodenum and colon after OVA challenge (Figure 2A,B). We observed that in OVA-induced allergic enteritis, MC numbers were strongly decreased in response to treatment with resveratrol. This observation was made in the colon (Figure 2B) as well as in the duodenum (Figure 2A). Thereby, a higher MC number was detected per mm² in the duodenum of the mice receiving OVA in direct comparison to colon sections. Exemplary pictures of MC in duodenum tissue of OVA-treated mice are shown in Figure 2C. Noteworthy, resveratrol diminished MC in both GIT sections down to a level of the control mice not receiving OVA (Figure 2A,B). Expression of MC proteases such as MCPT4 and MC-CPA was also strongly increased in intestinal tissues of OVA-treated mice (Figure 2D,E). However, we did not detect significant inhibitory effects of resveratrol on mRNA expression of MC proteases in comparison to the control group receiving the vehicle ethanol. In addition to increased MC numbers and increased expression of MC proteases, we found an increased expression of the receptor for the murine MC growth factor IL-3 [58] in OVA-induced enteritis. Remarkably, the expression of the α-chain of the IL-3 receptor was strongly down-regulated in response to treatment with resveratrol (Figure 2F). This finding can explain the inhibitory effect of resveratrol on MC in OVA enteritis.

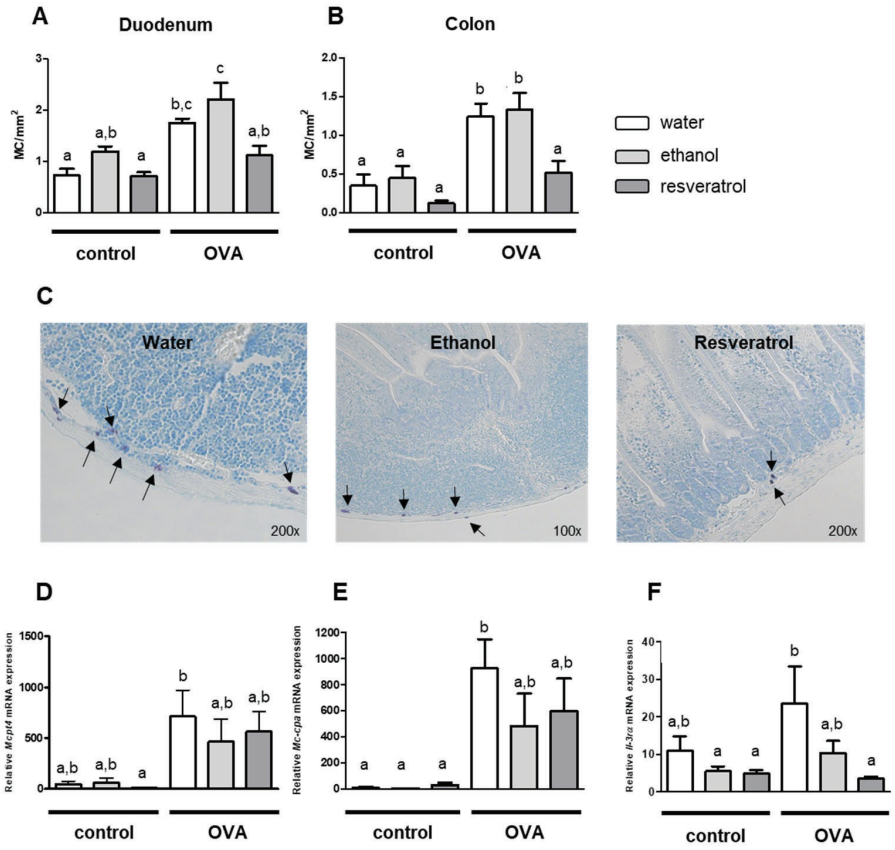

Figure 2. Mast cell (MC) numbers per mm² in duodenum (**A**) and colon (**B**) tissue and representative pictures of MC in duodenum of OVA-treated mice (**C**). mRNA expression of *Mcpt4* (**D**) (n = 8), *Mc-cpa* (**E**) (n = 5) and *Il-3rα* (**F**) (n = 6–8) in colon. Mice received water (white), 50 mg/kg BW ethanol (light grey) or 50 mg/kg BW resveratrol (dark grey) via drinking water for 28 days and were i.p. sensitized with 50 µg OVA in alum (1:2) on day 5 and 11 and further treated with or without (control) 50 mg OVA orally on day 15, 18, 20, 22, 24 and 27. Numbers of MC were determined in toluidine blue stained duodenum and colon sections, respectively and are indicated by black arrows (**C**), group size was n = 8, respectively. Values are mean ± SEM. Common letters indicate no significant difference between groups, different letters indicate significant change with at least $p < 0.05$. *p*-values for the respective data sets are given in Figure S2.

2.2. Resveratrol Inhibits Increase in MC and Shows Anti-Inflammatory Effects in IL-10$^{-/-}$ Mice

As increase in MC numbers in OVA-induced allergic enteritis was prevented by resveratrol application, we further aimed to check for resveratrol effects in experimental colitis of IL-10$^{-/-}$ mice. It was previously shown that MC are enhanced in patients' inflamed tissue suffering from IBD [30,31,50]. Moreover, Hamilton et al. supposed that MC tryptase could play a critical pro-inflammatory role in IBD [33]. We could show that, like in OVA-induced enteritis, an increase in MC numbers takes place in the duodenum and colon of IL-10$^{-/-}$ mice (Figure 3A,B). Again, treatment with resveratrol prevented the increase in MC numbers in duodenum and colon sections of IL-10$^{-/-}$ mice (Figure 3A,B). Colitis-related parameters such as tissue damage (Figure 3C), reduction in goblet cell numbers (Figure 3D) as well as immune cell infiltration (Figure 3E) in colon were significantly elevated in IL-10$^{-/-}$ mice and lowered by resveratrol treatment. In all cases, the scores of resveratrol receiving IL-10$^{-/-}$ mice did not significantly differ from the levels of the control wildtype groups. Overall, 6 IL-10$^{-/-}$ mice (40%) receiving ethanol as well as 4 animals (60%) receiving no additive developed signs of severe colitis and had to be removed from the study due to reaching a single end point score of 2 or a cumulative score

of ≥3. IL-10$^{-/-}$ group receiving resveratrol showed a similar survival rate of 60%, but it is noteworthy that onset of colitis and end point scores for these animals were achieved clearly later (day 64) in the experimental time course compared to control IL-10$^{-/-}$ groups receiving no additive (day 50) or ethanol (day 51) (Figure 3F).

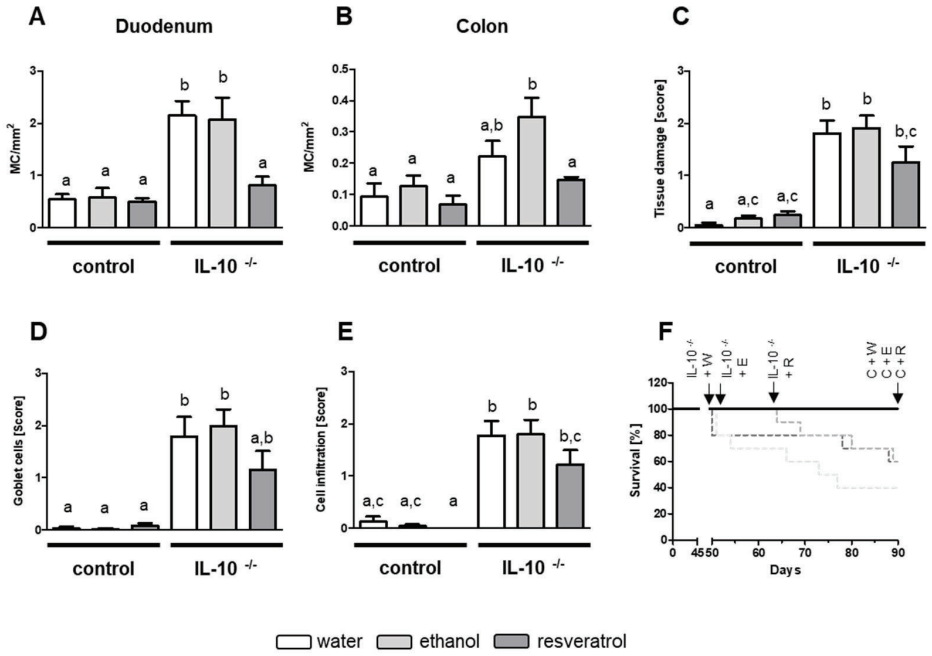

Figure 3. MC numbers per mm² in duodenum (**A**) and colon (**B**) tissue samples and scores for (**C**) tissue damage, (**D**) goblet cell numbers and (**E**) cell infiltration in colon as well as (**F**) survival rate [%] of mice after 90 days. Mice received water (white, (W)), 50 mg/kg BW ethanol (light grey, (E)) or 50 mg/kg BW resveratrol (dark grey, (R)) via drinking water for 90 days. Scores were determined in at least three H&E-stained tissue sections. MC numbers were counted in toluidine blue stained duodenum and colon sections, respectively. Group size was n = 5 for BALB/c mice (controls, (C)) and n = 9–10 for IL-10$^{-/-}$, respectively. Values are mean ± SEM. Common letters indicate no significant difference between groups, different letters indicate significant change with at least $p < 0.05$. *p*-values for the respective data sets are given in Figure S3.

2.3. Resveratrol Inhibits Degranulation and Expression of Pro-Inflammatory Cytokines in BMMC

We recently reported that resveratrol inhibits activation of hiMC [40]. As the increase in MC numbers was prevented in duodenum and colon of mice in OVA enteritis and IL-10$^{-/-}$ mice, we wanted to check for its effects on MC from murine origin. Thus, bone marrow-derived MC (BMMC) were incubated either with a concentration of 50 μM resveratrol prior to IgE-dependent stimulation or lipopolysaccharide (LPS) stimulation. Degranulation, measured as β-hexosaminidase release, was almost completely inhibited by resveratrol treatment in IgE-dependently activated BMMC (Figure 4A). Moreover, in IgE-activated BMMC mRNA expression of pro-inflammatory cytokines CCL2 (Figure 4B) and TNF-α (Figure 4C) were inhibited to the level of unstimulated controls. In addition, we observed strongly reduced mRNA expression of the cytokine TNF-α in response to treatment with resveratrol prior to stimulation with LPS (Figure 4D).

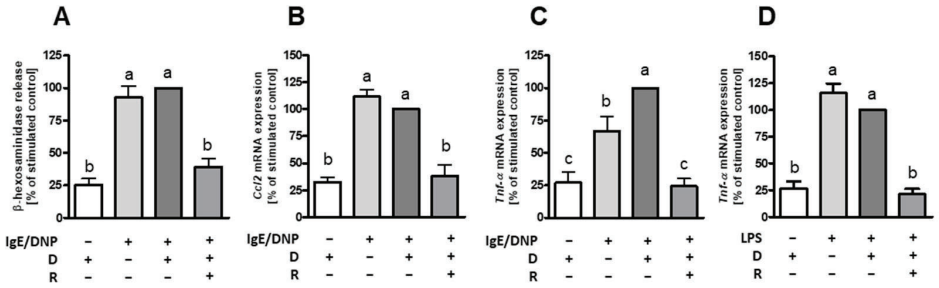

Figure 4. Degranulation, chemokine and cytokine mRNA expression in mouse bone marrow-derived mast cells (BMMC) following treatment with resveratrol. Cells were incubated with 50 μM resveratrol (R) or the corresponding vehicle control DMSO (D) for 60 min prior to 2,4-dinitrophenyl (DNP)-specific IgE treatment for 60 min and subsequent stimulation with 10 μg/mL DNP for 30 min to determine β-hexosaminidase release (**A**) (n = 14) and 90 min for mRNA expression of *Ccl2* (**B**) (n = 3) and *Tnf-α* (**C**) (n = 10). For lipopolysaccharide (LPS) stimulation, cells were incubated with 50 μM resveratrol or the corresponding control DMSO for 60 min prior to treatment with 1 μg/mL LPS for 3 h to determine *Tnf-α* mRNA expression (**D**) (n = 13). Values are mean ± SEM. Common letters indicate no significant difference between groups, different letters indicate significant change with at least $p < 0.05$. *p*-values for the respective data sets are given in Figure S4.

3. Discussion

In this study we could demonstrate that OVA-induced allergic Th2 immune response results in a strong increase in MC numbers in the GIT and that resveratrol treatment inhibits this increase in MC as well as the increased expression of the α-chain of the IL-3 receptor, the murine MC growth factor. We also found an increase in MC in experimental IL-10$^{-/-}$ colitis compared to wildtype mice, which, again, was diminished by resveratrol treatment. Together with our findings in vitro showing a strong inhibitory effect of resveratrol on MC activation, these data indicate that resveratrol may be considered as nutraceutical in the treatment of MC associated diseases such as allergies or IBD.

Histological changes observed in allergen-induced enteritis are combined with the infiltration of inflammatory cells into different layers of the intestinal wall [28,48,49]. We observed pronounced MC numbers in duodenum and colon of OVA-challenged BALB/c mice. This is in concordance with observations from Brandt et al. [28], who showed increased MC numbers in OVA-treated mice and that diarrhea was mediated by MC presence. Increased numbers of MC in affected tissue sections are reported by several studies examining food allergy [59,60] together with elevated levels of MC associated parameters such as MC protease 1 (MCPT1) [24,48,60] or histamine [24,57,61] in the respective intestinal areas or sera. We found that resveratrol treatment for 28 days inhibited the increased MC presence in both GIT sections duodenum and colon. In accordance with our data, resveratrol was shown to be able to prevent passive cutaneous anaphylaxis in mice [62] as well as onset of food allergy [47].

IL-3 is known to be the major growth factor for murine MC [58,63]. Moreover, binding of IL-3 to its receptor induces release of several cytokines such as CXCL8 [64]. IL-3R is also expressed in hiMC and hiMC growth is enhanced if cells were cultured with stem cell factor (SCF) together with IL-3. Enhanced histamine as well as leukotriene C4 (LTC4) release after FcεRI-crosslinking could also be detected [65]. We observed the enhancement of IL-3 receptor α chain (IL-3Rα) expression in OVA enteritis. This finding is in accordance with the increased MC numbers in OVA enteritis. Noteworthy, the increased expression of IL-3Rα was totally blocked by resveratrol. This finding strongly supports the assumption that MC numbers are regulated by the expression of the growth factor receptor and that resveratrol limits MC numbers by blocking IL-3Rα expression.

Elevated presence of MC may be accompanied with enhanced MC activity. The enhancement of proteases levels in food allergy was previously shown [48,57,66]. However, we did not find significant inhibitory effects of resveratrol on mRNA expression of MC

proteases in duodenum and colon sections, which have been strongly enhanced in OVA-challenged animals.

OVA is a common allergen to induce several experimental allergic reactions in mice [45–48,59,67], which can be accompanied by clinical symptoms such as weight loss in affected animals [31,48,68]. However, we did not observe significant effects on weight changes in OVA-treated mice compared to control mice (Figure S5). In mouse models of atopic dermatitis evaluating the role of resveratrol, differences in body weight gain also did not differ between the study groups [69,70]. Onset of diarrhea is one of the most described clinical symptoms in food allergy [28]. We observed stool softening after the 4th allergen challenge, but none of the animals showed signs of severe diarrhea throughout the whole experiment. Brandt and colleagues [28] described diarrhea after the 3rd and 4th OVA applications. From a total of 10 allergen challenges, Huang et al. observed diarrhea symptoms not before the 6th one [71,72]. In a study examining the role of coumarin in OVA anaphylaxis, diarrhea score increased with ongoing allergen challenges [66]. Thus, the absence of severe diarrhea in our experiments could be the result of the shorter treatment time and severe diarrhea may be appeared with ongoing OVA challenges and elongated experiment time.

Besides infiltration with inflammatory cells into affected tissues, food allergies are further associated with several histological changes in the intestine [48,55–57]. We observed that tissue damage, evaluated as epithelial barrier disruption, was slightly enhanced in intestinal tissues of OVA mice, with no significant attenuation by resveratrol. In contrast to OVA-induced enteritis, changes on histological levels were clearly detectable in IL-10$^{-/-}$ mice which develop a spontaneous form of chronic colitis due to the missing anti-inflammatory cytokine IL-10 [53]. We observed significantly increased epithelial damage, a reduced number of goblet cells and immune cell infiltration in all IL-10$^{-/-}$ groups as well as delayed onset of colitis symptoms. Treatment with resveratrol resulted in a down-regulation of these scores to levels not different from those of control wildtype animals. Moreover, resveratrol was also able to inhibit the increase in MC numbers in duodenum and colon tissue of colitis mice as found for MC numbers in OVA enteritis.

Although we observed strong inhibitory effects of resveratrol on the increase in MC counts, the effects on MC mediator release were less clear. Recently, we reported that resveratrol is a very potent inhibitor of hiMC. Pre-treatment with resveratrol almost abolished degranulation and expression of the cytokines CXCL8, CCL2, CCL3, CCL4 and TNF-α in hiMC in response to FcεRI receptor crosslinking [40]. Here, we examined the anti-inflammatory and anti-allergic effect of resveratrol on BMMC generated from wildtype mice. As found for human MC, resveratrol significantly diminished β-hexosaminidase release as well as gene expression of *Tnf-α* and *Ccl2* after IgE-mediated activation of BMMC. Similar observations of inhibitory effects of resveratrol on IgE-dependently activated MC were previously reported [39,73]. β-hexosaminidase or histamine release by MC may be affected by several signaling pathways initiated via FcεRI crosslinking. For example, studies already showed that resveratrol inhibits type II phosphatidylinositol (PtdIns) 4 kinase [74], phosphorylation of protein kinase C-μ (PKC-μ), phospholipase-γ (PLC-γ) and spleen tyrosine kinase Syk [62]. Additionally, ATP generated from oxidative phosphorylation in mitochondria was already shown to play an important role for MC degranulation [75] and that activated mitochondrial STAT3 as well as mitochondrial ERK1/2 are blocked by resveratrol [40].

In case of chronic inflammation and bacterial infections, the cell wall component of Gram-negative bacteria LPS is bound to CD14 membrane protein necessary for activation of MC via Toll-like receptor 4 (TLR4) which initiates cytokine and chemokine production in MC [76,77]. We were able to show that treatment with resveratrol totally blocked the mRNA expression of the pro-inflammatory cytokine TNF-α in both BMMC treated with LPS and BMMC stimulated by FcεRI receptor crosslinking. Li et al. [73] detected decreased TNF-α and IL-6 secretion in IgE mediated activated BMMC in response to a concentration of 10 μM resveratrol. Release of IL-6, TNF-α and IL-13 was reduced by more

than 50% by a concentration of 25 μM resveratrol in IgE-activated BMMC [21]. We found a complete inhibition of IgE-mediated mRNA expression of Tnf-α and Ccl2 in response to treatment with 50 μM resveratrol. CCL2 and TNF-α serve as regulatory factors in endothelial and immune regulation by attracting macrophages, neutrophils and basophils to inflammation sites and therefore promoting inflammatory reactions [78–80]. TNF-α as well as CCL2 are also relevant in pathogenesis of IBD as they are released by MC during early stages of inflammation and needed for sustaining colitis [81]. Inhibition of CCL2 pathway was shown to prevent a Th2 response in allergic asthma [82].

Not only resveratrol but also several other natural plant substances have been shown to have an anti-inflammatory effect on MC. In in vivo OVA models these were, amongst others, Chinese sweet tea (1% weight/volume (*w*/*v*)) [61], the flavonoid dihydromyricetin (10 mg/kg) [83], polyphenols from Arecae semen (0.1% *w*/*v*) [60] or the polyphenol fisetin (3 mg/kg/day) [84]. Degranulation of MC in jejuni (ca. 50%) together with reduction in MCPT1 (ca. 15%) and histamine levels (ca. 60%) of mice suffering from Th2-induced food allergy was further attenuated by polysaccharides from Aloe vera gel (100 mg/kg) [49]. We found that citrus flavonoids tangeretin and especially nobiletin affect activation of hiMC [85]. Moreover, we found that cinnamon extract, similar to resveratrol, was a more potent inhibitor of MC activation than the citrus flavonoids [40,51,52]. On the other side, cinnamon extract and nobiletin showed clearer effects on the attenuation of symptoms in the pathology of colitis in IL-10$^{-/-}$ mice than resveratrol [51,52,86]; nonetheless, similar to resveratrol, nobiletin also delayed the onset of symptoms in affected animals during the experimental course [52]. These observations lead to the assumption that nobiletin and cinnamon extract may be more auspicious substances than resveratrol in the treatment of colitis [51,52,87]. This may be due to the low bioavailability of resveratrol [38,88] which should be in the focus of future research. The poor bioavailability of resveratrol is due to its transformation into glucuronide and sulphate derivatives, both in liver and intestine [38]. It is important to note that most of the resveratrol is excreted unmetabolized (75%) and that the highest detected amount of free resveratrol was 1.7–1.9% [88]. Even though bioavailability of resveratrol or other polyphenols [89] seems to be a major problem in using them as nutraceutical, there are numerous studies reporting anti-inflammatory effects of resveratrol when applied either via oral gavage [24,46], chow [45,47] or as additive in drinking water [90,91]. Nevertheless, it seems to be necessary to increase the bioavailability of polyphenols, e.g., by encapsulation with carrier substances [46,92,93]. Regarding the application of resveratrol in humans, trials using concentration ranging from 10 mg to 5 g have already been carried out successfully [94–96]. Nonetheless, concentrations higher than 500 mg provoked mild to moderate gastrointestinal symptoms [96,97]. A concentration of 50 mg/kg BW in mice corresponds a dose of 243–324 mg in adult humans weighting 60–80 kg [98,99], respectively, and these concentrations are in the range of the concentrations commonly used for human trials and stated to be safe [96,97]. In addition, these levels are below the highest commercially available single dose of about 500 mg per tablet or capsule [100]. In vitro concentrations of resveratrol are also varying from low of 0.03 μM [20] to high doses of 100–200 μM [19], depending on the in vitro models used. The high metabolism and excretion of resveratrol [87] leads to low physiological concentrations (50 nM–2 μM), so that the in vitro concentrations used cannot be reached physiologically by the consumption of resveratrol in food [93].

In conclusion, our results demonstrate that resveratrol treatment prevents the increase in MC in allergen-induced Th2 enteritis as well as in experimental IL-10$^{-/-}$ colitis. Inhibitory effects of resveratrol on MC regarding degranulation and cytokine expression were also found in vitro. Therefore, resveratrol may be considered as an anti-allergic and anti-inflammatory plant-derived component for the prevention or treatment of MC-associated disorders.

4. Materials and Methods

4.1. Animals and Treatments

Four-week-old male BALB/c wild type mice (Janvier Labs, Le Genest-Saint-Isle, France) or IL-10 knockout mice (IL-10$^{-/-}$) with a BALB/c background were kept in a specific pathogen-free barrier (SPF) facility under controlled conditions and a light/dark cycle of 12 h accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were kept one mouse per cage and were provided a standard diet (ssniff Spezialdiäten GmbH, Soest, Germany) and drinking water ad libitum. All treatments and procedures were approved by the local Institutional Animal Care and Use Committee of the Ministry of Agriculture, Rural Areas, Veterinary and Food Sector of Stuttgart (permission number: V364/20; RPS35-9185.99/363, permission received on 24th June 2020). OVA enteritis model was as follows: mice were randomly divided into six groups (each n = 8), three for control and three for OVA treatment, receiving either no additive, 50 mg/kg body weight/d ethanol (≥99.8%; Carl Roth, Karlsruhe, Germany) or 50 mg/kg body weight/day resveratrol (dissolved in ≥99.8% ethanol; Carl Roth) in drinking water. Animals of the OVA groups were intraperitoneally (i.p.) sensitized with 50 µg OVA (Genaxxon bioscience, Ulm, Germany) in alum (1:2) (Thermo Fisher Scientific, Bonn, Germany) on challenge day 5 and 11. Oral gavage of 50 mg OVA dissolved in 250 µL 0.9% physiologic salt solution (NaCl; B. Braun, Melsungen, Germany) was carried out 6 times on challenge day 15, 18, 20, 22, 25, and 27. IL-10$^{-/-}$ colitis model: Mice were randomly divided into six groups, three groups consisting of BALB/c wildtype mice (each n = 5) and three groups of IL-10$^{-/-}$ mice (each n = 10). Thereby, animals received either no additive, 50 mg/kg body weight/day ethanol or 50 mg/kg body weight/day resveratrol (dissolved in ethanol) in drinking water for a total time span of 90 days. Drinking volume and body weight were determined every second day and quantity of ethanol or resveratrol was adjusted to the drinking volume of every mouse. Mice were anesthetized with 100 mg ketamine/kg body weight and 16 mg xylazine/kg body weight i.p. and sacrificed by cervical dislocation on experimental day 28 (OVA model) or day 90 (IL-10$^{-/-}$) when showing no previous signs of inflammation.

4.2. Assessment of OVA-Induced Allergic Enteritis and Colitis in IL-10$^{-/-}$ Mice

Severity of enteritis and colitis was monitored everyday based on a scoring system, accredited by the local ethics committee, with scores ranging from 0 to 3. Important end points of this scoring system were amongst others body weight change, as follows: 0: 0–1%, 1: 1–10%, 2: 10–20%, 3: ≥20%; rectal inflammation: 3: prolapse and/or rectal bleeding and consistency of stool: 3: diarrhea. Scoring of the stool followed the respective criteria, as follows: 0: normal, firm and round-formed feces; 1: soft and round-formed feces; 2: very soft and in parts unformed feces; 3: diarrhea. Mice were killed after reaching a single score of 2. When reaching a cumulative score of 3 or more, mice were killed after a 24 h observation period without recovery. Tissue samples of small intestine and colon were immediately fixed in 4% phosphate buffered formalin solution (Carl Roth) for later embedding or frozen immediately in liquid nitrogen for later isolation of RNA.

Intestinal inflammation was examined using formalin-fixed and paraffin-embedded (Carl Roth) samples of duodenum and colon stained with hematoxylin (Sigma Aldrich, Darmstadt, Germany) and eosin (Sigma Aldrich) (H&E) as previously described [51]. The scores ranged from 0 to 3 and contained the criteria for tissue injury (score 0: undamaged mucosa, 1: single lymphoepithelial damages, 2: surface damages of mucosa, 3: extensive mucosal damage and damage of deeper structures of the intestinal wall), number of goblet cells (score 0: normal; score 1: <50% reduction; score 2: 50–90% reduction; score 3: >90% reduction), and infiltration of inflammatory cells (score 0: low numbers of inflammatory cells in lamina propria; score 1: increased number of inflammatory cells in the lamina propria; score 2: accumulation of inflammatory cells in the lamina propria and infiltration

into the submucosa; 3: transmural distribution of inflammatory cells) (Figure S6). Further, bowel wall thickness was measured from muscularis externa to crypt base.

4.3. Histological Analysis of MC

Formalin-fixed tissue samples were embedded in paraffin. After deparaffinization and rehydration, 5 µM thick sections were stained with toluidine blue (Carl Roth) for visualization of MC as previously described [51,52]. Total MC number was obtained at 200–400x in the high field. Microscopic visualization of all parameters was conducted by usage of AxioVision software (Carl Zeiss Microscopy, Jena, Germany).

4.4. Generation of BMMC

Skin and muscles were removed from tibia and femur of wild type mice and DPBS (Gibco; Thermo Fisher Scientific) was used for rinsing out the bone marrow cells. Cells were counted and suspended in 90% fetal calf serum (FCS; Merck; Darmstadt, Germany) with 10% DMSO (Carl Roth) and frozen in liquid nitrogen until further processing. Bone marrow cells were cultured in an overall volume of 5 mL BMMC medium (RPMI1640 GlutaMaxTM (Gibco; Thermo Fisher Scientific) with 10% FCS, 1% penicillin-streptomycin solution (HyCloneTM Laboratories, South Logan, USA) in the presence of murine IL-3 with a final concentration of 30 ng/mL (Peprotech, Hamburg, Germany). During the first 5 weeks of cultivation, medium and plates were changed twice a week, then medium was changed once a week to remove adherent cells. Suspension cells increased in size and developed a round shape. After culturing for 6 to 8 weeks the cells were used for functional assays. Maturity and purity of the BMMC were examined on cytospins stained with May-Grünwald/Giemsa (Carl Roth, medite histotechnic, Burgdorf, Germany).

4.5. Treatment of BMMC

Cells were treated with 50 µM resveratrol (Sigma Aldrich, St. Louis, MO, USA) 1 h prior to incubation with IgE-specific 2,4-dinitrophenol (IgE-DNP; provided by U. Blank, French Institute of Health and Medical Research, Paris, France) for 90 min at 37 °C. Cells were washed twice with DPBS and stimulated with 10 µg/mL DNP (Thermo Fisher Scientific) for 90 min at 37 °C to analyze mRNA expression or 30 min to determine β-hexosaminidase release. For LPS stimulation, cells were treated with 50 µM resveratrol for 1 h prior to 1 µg/mL LPS (Escherichia coli O111:B4; Sigma Aldrich) stimulation for 3 h. Unstimulated controls contained the same concentrations of the vehicle DMSO.

4.6. Measurement of Degranulation

Degranulation of BMMC was measured by determining the amount of released β-hexosaminidase in supernatants by a color enzyme assay [101]. In brief, cell supernatants were incubated with 50 µL of 4-nitrophenyl-*N*-acetyl-β-D-glucosamid (pNAG; Carl Roth) for 1h hour at 37 °C. The enzymatic conversion of pNAG by β-hexosaminidase into 4-nitrophenol was stopped with 150 µL of 0.2 glycine (pH 10.7; Carl Roth). β-hexosaminidase release was measured by its enzymatic 4-nitrophenol product in a photometer at 405 nm wavelength.

4.7. RNA Preparation and Real-Time RT-PCR

Total RNA was obtained from cell lysates using EXTRACTME® TOTAL RNA kit (blirt, Gdansk, Poland) and from tissue samples using peqGold TrifastTM (VWR International GmbH, Erlangen, Germany). Real-time RT-PCR reactions were performed in optical tubes containing cDNA template, each sense and anti-sense primer, and SsoFastTM EVAGreen Supermix (Bio-Rad Laboratories, Feldkirchen, Germany). Reaction mixture without cDNA was used as negative control. Relative quantification ($2^{-\Delta\Delta CT}$) was performed using glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) housekeeping gene as reference. Mouse sense and antisense primer sequences were as follows: *Gapdh*: 5′-TGT

TCC TAC CCC CAA TGT GT-3′, 5′-AGA GTG GGA GTT GCT GTT GA-3′, product size: 175 bp; *Ccl2*: 5′-ACT CAC CTG CTG CTA CTC AT-3′, 5′-TCA GCA CAG ACC TCT CTC TT-3′, product size: 138 bp; *Il-3rα*: 5′-TGG AGG AAG TCG CTG CTC TA-3′, 5′-CGT CAC CTC GCA GTC TTC AA-3′, product size: 111 bp; *Tnf-α*: 5′-ACC ACC ATC AAG GAC TCA-3′, 5′-AGG TCT GAA GGT AGG AAG G-3′, product size: 127 bp; *Mc-cpa*: 5′-CAT GGA CAC AGG ATC GAA TG-3′, 5′-TGC AGG TCC CCT GTA GAC AT-3′, product size: 152 bp; *Mcpt4*: 5′-ATC TTA TGG ACG CGG AGA TG-3′, 5′-GTG ACA GGA TGG ACA CAT GC-3′, product size: 185 bp; (all Eurofins Genomics, Ebersberg, Germany). The CFX 2.1 software and CFX Connect Real-Time PCR System of Bio-Rad Laboratories were used.

4.8. Statistics

Data are expressed as mean ± standard error of the mean (SEM). If not stated otherwise, student's *t*-test was used for differences in in vitro experiments and one-way analysis of variance (ANOVA) with Tukey's post hoc test was used to analyze differences between treatment groups in in vivo experiments. Statistically significant differences between treatment groups are shown by different letters. Common letters between treatment groups mean no significant difference. A value of $p < 0.05$ is considered to be statistically significant. GraphPad Prism scientific software version 5.0 (San Diego, CA, USA) was used for statistical analysis.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/ijms23031213/s1.

Author Contributions: Conceptualization, A.L.; investigation, S.B. and A.L.; data analysis, S.B., J.A., N.S., M.F. and A.L.; writing—original draft preparation, S.B; writing—review and editing, A.L.; supervision, A.L.; project administration, A.L.; funding acquisition, A.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by German-Israeli Foundation for Scientific Research and Development (GIF) (Grant No.: I-1469-414.13/2018).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All treatments and procedures were approved by the local Institutional Animal Care and Use Committee of the Ministry of Agriculture, Rural Areas, Veterinary and Food Sector of Stuttgart (permission number: V364/20; RPS35-9185.99/363, permission received on 24th June 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available on request from the authors.

Acknowledgments: The authors thank Yvonne Soltow for excellent technical assistance. The authors also thank the staff of the central facility for biological and biomedical research with laboratory animal husbandry at the University of Hohenheim for excellent animal care.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Effects of resveratrol on mast cells and its potential use as nutraceutical – overview of current studies

Since MC are the main effector cells of allergy, we wanted to give a brief summary of *in vitro* as well as *in vivo* studies examining the role of resveratrol application on MC associated allergic reactions. Natural substances are increasingly becoming the focus of scientific attention, because they could be used as nutraceuticals due to their positive properties [110].

Aim of this overview was to summarize studies that examined the effects of resveratrol on MC *in vitro* and *in vivo*. Since degranulation of MC is the most prominent and common trigger of MC associated inflammatory reactions and symptoms, all factors contributing to degranulation as cause or result may be of interest when looking for possible effects of resveratrol. Parameters we considered important here were degranulation, arachidonic acid metabolites, cytokines and chemokines as well as focus on MC signaling. Additionally, we reviewed on a relative sparse number of randomized controlled trials examining allergic rhinitis that looked after a beneficial role of resveratrol [130, 131]. The use of substances of natural origin as a nutraceutical is further dependent on their bioavailability [132, 133]. Challenges may occur if the substance of interest has low bioavailability, which is the case for resveratrol [134]. To overcome the problems of low bioavailability, numerous methods are available to date [135]. Nonetheless, there are various study problems related to the concentration of the substance used or administered especially when using polyphenols such as resveratrol, [136, 137].

We report that resveratrol has a wide range of immunomodulatory effects in *in vitro* and *in vivo* MC mediated allergic reactions. Further, we clarify that there are challenges encountered due to the low bioavailability of this polyphenol and present methods to overcome this problem of using resveratrol as nutraceutical.

The article was published 2022 in “Molecular Nutrition and Food Research” [22].

REVIEW

Molecular Nutrition & Food Research
www.mnf-journal.com

Resveratrol Attenuates Mast Cell Mediated Allergic Reactions: Potential for Use as a Nutraceutical in Allergic Diseases?

*Mehtap Civelek, Sabrina Bilotta, and Axel Lorentz**

Allergic diseases are one of the most common health disorders affecting about 30% of the world population. Mast cells (MCs) are key effector cells of allergic reactions by releasing proinflammatory mediators including histamine, lipid mediators, and cytokines/chemokines. Natural substances like secondary plant substances such as resveratrol (RESV), which can contribute to prevention and treatment of diseases, are becoming increasingly interesting for use as nutraceuticals. In this review, the anti-inflammatory effects of RESV on MC-mediated allergic reactions in vitro and in vivo models are summarized. The studies indicate that RESV inhibits MC degranulation, synthesis of arachidonic acid metabolites, expression of cytokines and chemokines as well as activation of signal molecules involved in proinflammatory mechanisms. Also, beneficial impacts by this polyphenol are reported in randomized controlled trials with allergic rhinitis patients. Although it cannot yet be concluded that RESV can be used successfully in allergy patients in general, there are many results that indicate a possible role for RESV for use as an anti-inflammatory nutraceutical. However, strategies to favorably influence the poor bioavailability of RESV would be helpful.

1. Introduction

The prevalence of allergic diseases is high; almost 30% of the world population suffers from one or more allergic conditions.[1] In general, allergy is an overreaction of the immune system to ordinarily harmless foreign substances, usually proteins, resulting in skin rash, sneezing, or swelling of mucous membranes.[2] Therefore, the term "allergy", which was discovered by Clemens von Pirquet (1874–1929) in the early 1900s, describes a constellation of clinical diseases like allergic rhinitis (AR), asthma, atopic dermatitis (AD), food allergy, drug allergy, and the life threatening systemic mast cell (MC)-mediated reaction known as anaphylaxis.[3] MCs belong to the innate immune system. They play a crucial role in inflammatory and immediate allergic reactions by releasing inflammatory mediators such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid (AAM) that act among others on inflammatory cells.[4] Noteworthy, MC granules are described as the major source of histamine in humans.[3] The so-called degranulation can be induced by exogenous and endogenous stimuli, including immune mechanisms that may be Immunglobulin (Ig) E-dependent or IgE-independent.[4] IgE antibodies produced in response to a certain allergen bind to the high-affinity FcεRI receptor, expressed on the surface of MCs, and lead to their activation after they have been cross-linked by the allergen,[4,5] which is the so-called type I hypersensitivity allergic reaction.[6] Besides, interleukin (IL-) 33 released by exposure to allergens seems to play a role in IgE-dependent and -independent allergic inflammation via its receptor interleukin 1 receptor-like 1 (ST2).[7] IgE-mediated activation results in degranulation of preformed mediators such as proteases, and histamine or the de novo production of lipid mediators as well as cytokines influencing vascular permeability and adhesiveness.[4,8] Therefore, MCs are involved in several diseases such as allergic rhinitis, atopic dermatitis, asthma, but also in autoimmune disorders, atherosclerosis or mastocytosis.[8] **Figure 1** shows MC activation via several stimuli leading to the release of de novo and prestored mediators implicated in several diseases.

Since there is an increase in life expectancy all around the world,[9] there is a growing interest in substances, which can contribute to healthy aging by preventing diseases or treating existing disorders. In this context, in the late 1980s the term nutraceutical, consisting of "nutrition" and "pharmaceutical", was introduced for food or food components that are praised for maintaining health.[10] As the term implies, it is assumed that they exhibit pharmaceutical benefit besides their nutritional value.[11] Nowadays, different types of nutraceuticals are available. They can be categorized based on the food sources in probiotics/prebiotics, polyunsaturated fatty acids, antioxidant vitamins, spices, and polyphenols.[10] Latter comprise a large group of phenolic

M. Civelek, S. Bilotta, A. Lorentz
Institute of Nutritional Medicine
University of Hohenheim
70599 Stuttgart, Germany
E-mail: lorentz@uni-hohenheim.de

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202200170

DOI: 10.1002/mnfr.202200170

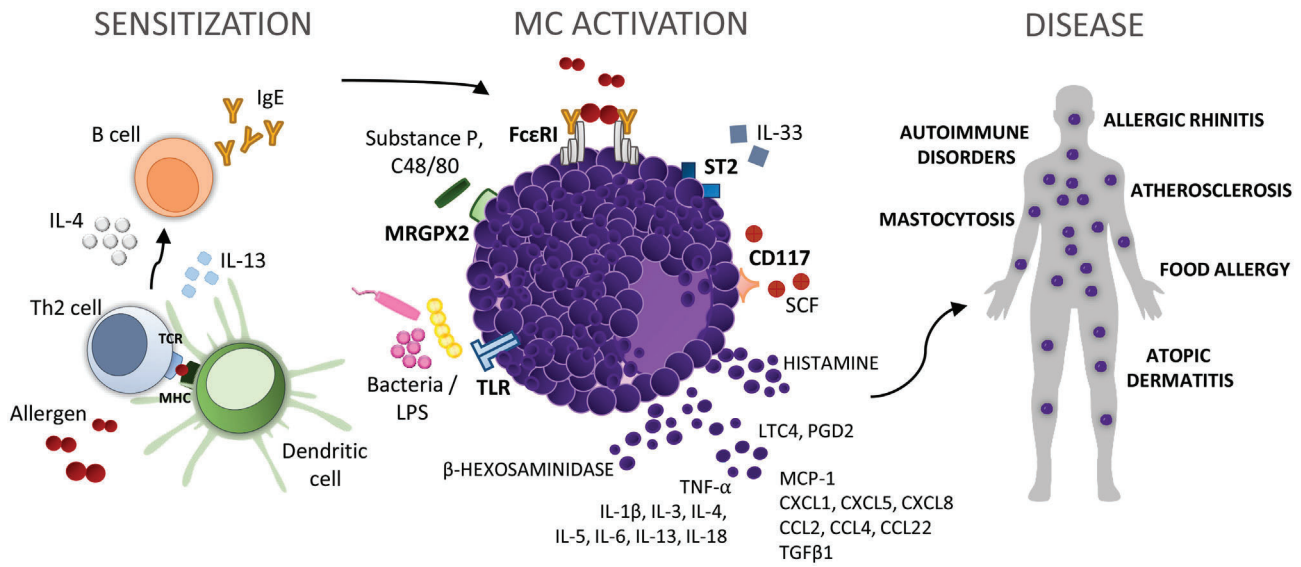

Figure 1. MC activation via several stimuli leads to release of de novo and prestored mediators involved in several diseases. Sensitization and subsequent FcεRI cross-linking represent the most prominent activation cascade in MCs. Antigen presenting dendritic cells present allergens via major histocompatibility complex (MHC) and interact with T cell receptor (TCR). Th2 cell derived IL-4 and IL-13 stimulate B cells to produce IgE. IgE then binds to FcεRI receptors on MCs. If allergens bind to specific IgE, FcεRI is cross-linked, leading to MC activation. Further, activation signals can be initiated via stimuli like substance P or compound 48/80 binding to Mas-related G-protein coupled receptor member X2 (MRGPRX2), bacterial components like LPS to toll-like receptors (TLR), IL-33 to ST2 receptor or SCF to CD117. Activation cascade leads to degranulation of prestored substances like histamine or β-hexosaminidase or de novo synthesis of cytokines/chemokines. Inflammatory mediators are involved in several disease outcome, e.g., allergic rhinitis, atopic dermatitis, food allergy, mastocytosis, atherosclerosis, or autoimmune disorders.

compounds, including flavonols, flavones, anthocyanins, coumarins, or stilbenes.[10,12]

Resveratrol (trans-3,5,4′-trihydroxystilbene) (RESV) belongs to the best studied polyphenols, precisely to the group of stilbenes. Its two phenol rings are connected by an ethylene bridge.[13] Just like other secondary plant substances, RESV is synthetized to defend plants against bacterial or fungal infection or external stress, including UV irradiation.[14,15] Naturally, RESV occurs as cis and trans isoforms in >70 plant species as well as different fruits, including blueberries, mulberries, raspberries, or grapes.[14] Nevertheless, it is mainly found in grape skin, at a concentration of 50–100 μg g^{-1}.[16] However, trans-RESV exists in glycosylated form and therefore is more stable which is why it is considered to be the most abundant form.[14] As an aglycone, trans-RESV has 38% bioavailability and its exposure was approximately 46-fold lower than that of the glucuronide form.[14] In this context, oral intake of 25 mg RESV resulted in a concentration peak of <10 ng mL^{-1} after 0.5 h.[15] The poor in vivo bioavailability of RESV is explained by its rapid metabolism to glucuronide and sulfate derivatives in the liver and intestine.[14] After RESV was discovered from white squash in the 1940s,[17] its possible beneficial effects have been examined in various studies. Therefore, RESV is probably best known for its antioxidant activities.[18] Also, anti-inflammatory,[19] antiallergic,[20] anticancerogenic,[21] cardio-,[22] and neuroprotective[23] or antipathogenic[24] effects of this polyphenol were shown in vitro and in vivo. It is even considered to mimic some aspects of caloric restriction,[25] which can extend lifespan.[26] Furthermore, clinical trials reported that RESV is safe and well tolerated.[27] Here, we summarize the immunomodulating activities of RESV on MCs in vitro and in vivo in the context of allergic conditions.

2. RESV Inhibits MC Degranulation In vitro and In vivo

MCs are located in mucosal and epithelial tissues where antigens can enter the host's body, such as the gastrointestinal tract, skin, or the respiratory epithelium.[28] Their cytoplasm contain about 50–200 large granules with preformed and stored inflammatory mediators as mentioned above.[28] In various in vitro as well as in vivo models, it could be shown that treatment with RESV inhibits MC degranulation (**Tables 1** and **2**). Determination of β-hexosaminidase (β-Hex) is used to evaluate the level of MC degranulation,[29] which can also be evaluated by detection of histamine. However, β-Hex release is slower and the process persists for longer than histamine release does.[29] In the rat basophilic leukemia mast cell line (RBL-2H3), which is widely used to study the IgE-dependent degranulation of MCs,[29] the release of β-Hex and/or histamine could be reduced by about 50% and more after the treatment with RESV (Table 1).[20,30,31] Naveen et al.[31] explained the RESV-induced decrease of β-Hex release by the inhibition of the type II phosphatidylinositol (Ptdlns) 4-kinase, which is usually activated upon FcεRI cross-linking.

Using mouse bone marrow-derived mast cells (BMMC-), Baolin et al.[32] could detect an inhibition of IgE-mediated histamine release by RESV at concentration of 100 μM without any cytotoxic effects. This reduction was greater than 90% (Table 1). However, lower doses did not result in a significant decrease.[32] RESV also attenuated IgE/antigen-mediated release of β-Hex by mouse BMMC.[33] Wang et al.[34] reported a dose-dependent attenuation of compound 48/80 (C48/80)-induced β-Hex and histamine release by RESV in the human Laboratory of Allergic Disease 2 (LAD2) mast cell line. Here, the application of 200 μM

Table 1. Overview of the anti-inflammatory effects of RESV on MCs in vitro.

Dosage RESV	MC model	Stimulus	Degranulation	Arachidonic acid metabolites	Cytokines/Chemokines (Gene Expression and/or Protein Level)	Signaling	Others	Reference
N/A	RBL-2H3	IgE/DNP-HSA	β-Hex ↓ (60%)	—	—	PtdIns 4-kinase activity ↓ (90%)		[31]
1–25 μM	RBL-2H3	Anti-DNP/DNP-HSA	—	—	TNF-α, IL-4, IL-3 ↓ (dd)	p-p38 MAPK, p-ERK1/2, p-JNK & p-Src ↓ (dd)	mRNA expression *Fcεriγ* subunit ↓ (dd)	[42]
10 μM	RBL-2H3	DNP-BSA	β-Hex ↓		—	p-PLCγ1 and p-ERK1/2 ↓ (N/A)	—	[65]
10 μM	RBL-2H3	IL-33 (50 ng mL^{-1}) and IgE-antigen	—	—	IL-6, IL-13, TNF-α, and MCP-1 ↓	ST2, cytosolic pERK1/2/ERK, pJNK/JNK ↔ Cytosolic p-P38/P38, IκBα, and NF-κB (p65) proteins ↓ (≈50–60%)	Cell viability ↓ (dd)	[43]
10 μM	RBL-2H3	AHR ligands/ Iono-PMA	β-Hex ↓ (<50%)	—	*Il-6* ↓	—	—	[30]
5, 10, and 20 μg mL^{-1}	RBL-2H3	Anti-DNP-IgE/DNP-BSA	β-Hex ↓ (dd; >50% with ≥10 μM)					[20]
≤25 mmol L^{-1}	RBL-2H3	Anti-DNPIgE/DNPHSA	β-Hex ↓ (dd) Histamine ↓ (dd)			p-PKCμ and p-PKCθ↓ p-PKCζ/λ↑ p-Syk & p-PLCγ↓		[38]
250	rPMC	C48/80	Histamine ↓ (82.4%)					[66]
1, 10, and 100 μM	mBMMC	IgE or Calcium ionophore A23187	Histamine ↓ (>90% with 100 μM)	LT ↓ (99.4% with 100 μM; 72% with 10 μM) PGD_2 ↓ (≈33% with 10 μM; ≈70% with 100 μM)	—	—	—	[32]
1–25 μM	mBMMC	IL-33 and anti-DNP-IgE/anti-IgE	CD63 counts ↓ (≈70% with 25 μM)	—	IL-6, IL-13, and TNF-α ↓ (dd; ≈20–30% with 10 μM, ≈40–50% with 25 μM)	p-IKKα/β & p-p65 ↔ p-p38 & p-MK2 ↔ p-Akt ↓	—	[7]
10 μM	mBMMC	Anti-DNP-IgE/DNP-HSA	β-Hex ↓ (≈65%)	LTC_4 and PGD_2 ↓ (80%)	IL-6 and TNF-α ↓ (≈70%)	p-Akt, p-p38, p-Syk, and p-PTP1B ↓	Intracellular Ca^{2+} ↓ (≈50%)	[33]
0.03, 0.3, and 3 μM	HMC-1	PMA + calcium ionophore A23187	—	—	TSLP ↓ (dd; ≈25% with 3 μM) *TSLP* ↓ (dd; ≈80% with 3 μM)	RIP2 ↓ (≈37% with 3 μM) and caspase-1 ↓ (≈60% with 3 μM) NF-κB ↓ (≈50% with 3 μM) and p-IκBα ↓ (≈30% with 3 μM)	Intracellular Ca^{2+} ↓ (≈30%)	[46]

(*Continued*)

Table 1. (Continued).

Dosage RESV	MC model	Stimulus	Degranulation	Arachidonic acid metabolites	Cytokines/Chemokines (Gene Expression and/or Protein Level)	Signaling	Others	Reference
10–50 µM	HMC-1	PMA + calcium ionophore A23187	—	—	*TNF*, *IL-6*, *IL-8* ↓ (≈65–90% with 50 µM) TNF-α, IL-6 & IL-8 ↓ (≈60–95% with 50 µM) —	*Cox-2* ↓ (≈80% with 50 µM) COX-2 ↓ (≈90% with 50 µM) p-ERK1/2/ERK1/2 ↓ (≈66% with 50 µM) and NF-κB activity ↓ (≈66% with 50 µM) degradation of IκBα ↓	Intracellular Ca^{2+} ↓ (≈80%)	[47]
50 µM	HMC-1	RESV &/or tocopherols	—	—		p-Akt ↓ (58%)	Cell proliferation ↓ (25% at 24 h; 49% at 48 h, and 39% at 72 h)	[67]
50, 100, and 200 µM	LAD2	C48/80	β-Hex ↓ (dd) Histamine ↓ (dd)	PGD_2 ↓	*MCP-1* ↓ (≈40%), *TNF* ↓ (≈60%), and *Il-1β* ↓ (≈80%) TNF-α, IL-8, and MCP-1 ↓ (dd)	*Nrf2*, *Ho-1* & *Nqo-1* ↑ (50–100%)	Intracellular Ca^{2+} (dd) ↓ *Mrgprx2* ↓ (≈50%)	[34]
50 µM	hiMC	mAb 22E7 (IgE-dependent activation)	β-Hex ↓ (dd; 75% with 50 µM)	—	*CXCL8*, *CCL2*, *CCL4* & *TNF* ↓ (dd; ≈80–100%) and *CCL3* ↓ (dd; ≈100%)	p-STAT3 & p-ERK1/2 in nuclear (≈50–70%) and mitochondrial fractions (≈60–85%) ↓	—	[35]

β-Hex, β-hexosaminidase; AHR, aryl hydrocarbon receptor; Akt, protein kinase B; BSA, bovine serum albumin; C48/80, compound 48/80; Ccl2, CC-chemokine ligand 2; COX-2, cyclooxygenase-2; Cxcl8, chemokine (C-X-C motif) ligand 8; dd, dose dependent; DNP, Dinitrophenol; ERK1/2, extracellular signal-regulated kinase 1/2 (ERK1/2); hiMC, human intestinal mast cells; HMC-1, human mast cell line 1; HSA, human serum albumin; HO-1, heme oxygenase 1; IgE, Immunoglobulin E; IL, Interleukin; IkBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IKKα/β, inhibitor of nuclear factor kappa-B kinase subunit alpha/beta; JNK, c-Jun N-terminal kinase; LAD2, Laboratory of Allergic Diseases 2; LT (C4), leukotriene (C4); mAb, monoclonal antibody; mBMMC-, (mouse)bone marrow-derived mouse mast cells; MC, mast cell; MCP-1, monocyte chemoattractant protein-1; MK-2, mitogen-activated protein kinase-activated protein kinase 2; MRGPRX2, MAS Related GPR Family Member X2; N/A, not available; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NQO1, NAD(P)H quinone oxidoreductase-1; Nrf2, nuclear factor erythroid 2-related factor 2; p, phospho; p38 MAPK, p38 mitogen-activated protein kinase; PGD2, prostaglandin D2; PKC, protein kinase C; PLCg1, phospholipase C, gamma 1; PLCγ, phospholipase Cγ; PMA, phorbol myristate acetate; PtdIns 4, Phosphatidylinositol 4-phosphate; PTP1B, protein-tyrosine phosphatase 1B; RBL-2H3, rat basophilic leukemia cells; RESV, resveratrol; RIP2, receptor-interacting protein 2; rPMC, rat peritoneal mast cells; ST2, Interleukin 1 receptor-like 1; STAT3, signal transducer and activator of transcription 3; Syk, spleen tyrosine kinase; TNF-α, tumor necrosis factor, alpha; TSLP, thymic stromal lymphopoietin.

Table 2. Overview of the anti-inflammatory effects of RESV on MCs in vivo.

Dosage RESV	MC model	Stimulus	Degranulation	Arachidonic acid metabolites	Cytokines/Chemokines (Gene Expression and/or Protein Level)	Signaling	Others	Reference
5 mg kg^{-1}	♂ Sprague Dawley rats (6 wk)	IL-33	—	—	Plasma levels ↓IL-6 (≈50%), IL-13 (≈40%), TNF-α (≈60%) and MCP-1 (≈50%)	—	—	[43]
15 mg kg^{-1}	♂ Sprague Dawley rats (adult)	IIR	Intestinal β-Hex ↓ (≈50%)	—	Serum levels ↓: TNF-α (≈50%), IL-1β (≈40%) & IL-18 (≈50%) *Il-1β p17* ↓ (≈60%) & *Il-18* ↓ (≈60%)	Mucosal NLRP3 & caspase-1 p20 ↓ (≈50%) Mucosal IL-1β p17 & IL-18 ↓ (≈60%)	TUNEL positive cells ↓ (≈50%)	[39]
0.5 and 5 mg kg^{-1}	(N/A) BALB/c mice (4 wk)	CRSwNP (OVA-induced)	—	PGDs↓ (≈80% with 0.5 mg kg^{-1} and 5 mg kg^{-1}) LTC_4s↓(≈75% with 0.5 mg kg^{-1}, ≈50% with 5 mg kg^{-1})	*Il-4*↓ (≈40% with 0.5 mg kg^{-1}, ≈60% with 5 mg kg^{-1}) & *Il-5*↓ (≈80% with 0.5 mg kg^{-1} and 5 mg kg^{-1})	5-LO↓ COX-2↓ (only with 5 mg kg^{-1})	—	[40]
10 mg kg^{-1}	♂ BALB/c mice (5 wk)	PCA (anti-DNP IgE / DNP-HSA)	MC degranulation in dorsal skin↓ Plasma histamine↓ (≈50%)	—	MCP-1↓ (≈50%) & MIP-2 (≈40%) in dorsal dermis	p-Syk ↓ (≈50%) p-PLC-γ ↓ (≈60%) p-PKC-μ ↓ (≈55%) in dorsal skin tissue	Vascular permeability (≈75%) and thickness of ears (≈50%) ↓	[38]
12.5 mg kg^{-1}	♀ BALB/c mice (6 wk)	Chronic allergic airway disease (OVA-induced)	—	—	TGFβ1 in lung tissue ↓	—	Total and differential BAL cell counts ↔ Inflammatory cell infiltration in airways ↓ (≈15%) subepithelial thickness of ECM ↓ (≈20%)	[49]
5, 10, and 20 mg kg^{-1}	♀ BALB/c mice (7–9 wk)	FA (OVA-induced)	Serum IgE ↓ (≈30%, ≈40%, ≈40%, respectively) Serum histamine ↓ (≈25% with 10 mg kg^{-1}, ≈50% with 20 mg kg^{-1})	—	Serum MCP-1↓ (≈30% with 10 mg kg^{-1}, ≈50% with 20 mg kg^{-1})	—	DC number in SPL ↓ (≈45% with 20 mg kg^{-1}) Th & Treg cells in SPL & MLN ↔ B cell number ↓ (≈20% in SPL, ≈25% in MLN) MC number ↓ (≈20% in SPL, ≈60% in MLN)	[20]
30 mg kg^{-1}	♀ BALB/c mice (6–8 wk)	AD (DNFB-induced)	—	—	Number of IL-15-, IL-33-, and TSLP-positive cells in skin epithelium ↓ (≈20%, respectively)	Number of caspase-3 positive cells in skin epithelium ↓ (≈25%)	Weight change ↔ Dermatitis score ↓ (≈40%) epithelial thickness ↓ (≈50%)	[68]

(*Continued*)

Table 2. (Continued).

Dosage RESV	MC model	Stimulus	Degranulation	Arachidonic acid metabolites	Cytokines/Chemokines (Gene Expression and/or Protein Level)	Signaling	Others	Reference
10 and 50 mg kg^{-1}	♀ BALB/c mice (7 wk)	AI (OVA-induced)	—	—	IL-4 ↓ (ns; ≈50%), IL-5 ↓ (≈60%; ≈40%), IL-13 ↓ (≈30%; ≈50%), and TGFβ1 ↓ (ns; ≈50%) in BAL fluid *Tgfβ1* in lungs ↓ (ns; ≈70%) TGFβ1 in lungs ↓ (ns; ≈60%) & BAL fluid ↓ (ns; ≈20%)	p-smad2 ↓ (≈30%, ≈90%) and p-smad3 in lungs ↓ (ns; ns)	Total cell counts ↓ (≈40%, respectively) and eosinophils ↓ (≈60%; ≈70%) in BAL fluid; Infiltration of peribronchial inflammatory cells in lungs ↓ Number of goblet cells ↓ (≈40%; ≈60%) α-SMA in peribronchium ↓ (≈20%; ≈40%) Hydroxyproline in lungs ↓ (ns; ≈60%)	[69]
5, 10, and 20 mg kg^{-1}	♂ C65BL/6 (8 wk old)	Pseudo-allergy (C48/80-induced)	Serum histamine ↓ (dd; ≈60%; ≈60%; ≈70%) Degranulated MC number ↓ (dd; ns; ≈50%; ≈70%)	—	Serum MCP-1↓ (≈50%, ≈70%, ≈80%) TNF-α↓ (≈30%, ≈50%, ≈70%) IL-8↓ (≈20%, ≈30%, ≈50%)	—	Paw thickness ↓ (dd; ≈20%; ≈60%; ≈60%) Evans blue extravasation ↓ (dd; ≈20%; ≈30%; ≈50%)	[34]
10 and 20 mg kg^{-1}	(N/A) C65BL/6 (NA)	Gouty arthritis (MSU-induced)	—	—	Release in joint tissue ↓: MCP-1 (≈100%), IL-1β (≈80%), IL-1α (≈90%), IL-6 (≈90%), TNF-α (≈100%), IFN-γ (≈100%), CXCL-1 (≈100%), CXCL-5 (≈90%), CCL-22 (≈70%) and CXCL-13 ↑ (≈400%)	*Sirt1* ↑ (≈100%) and *Ppary* ↑ (≈60%) in joint tissue ↑	Foot swelling ↓ Inflammation scores ↓ (≈100%) Infiltration of inflammatory cells in joints ↓	[70]
20 mg kg^{-1}	♀NC/Nga mice (6 wk old)	AD (DfE-cream induced)	MC number in skin↓ (≈10%)	—	Protein expression in skin ↓: TNF-α (≈60%), IL-1β (≈60%) HMGB-1 (≈70%) Serum IL-4 ↓ (≈70%) & IFN-γ (≈50%)	Protein expression in skin ↓: p-PI3K (≈70%), p-ERK1/2 (≈40%) & p-NF-κB (≈70%)	Dermatitis score ↓ Protein expression in skin ↓: TNFR1 (≈50%), IL-2Rα (≈70%), COX-2 (≈60%) & GRP78 (≈60%) CHOP (≈80%), cleaved caspase-7 (≈40%), TLR4 (ns), RAGE (≈40%)	[51]

♂: male; ♀; female; 5-LO, 5-lipoxygenase; α-SMA, alpha-smooth muscle actin; AD: atopic dermatitis; AI: airway inflammation; BAL, bronchoalveolar lavage; β-Hex, β-hexosaminidase; C48/80, compound 48/80; Ccl2, CC-chemokine ligand 2; CHOP, C/EBP homologous protein; COX-2, cyclooxygenase-2; CRSwNP, chronic rhinosinusitis with nasal polyps; Cxcl, chemokine (C-X-C motif) ligand; dd, dose dependent; DC, dendritic cells; DfE, *Dermatophagoides farinae*; DNFB, 1-Fluoro-2,4-dinitrobenzene; DNP, Dinitrophenol; ECM, extracellular matrix; ERK1/2, extracellular signal-regulated kinase 1/2; FA, food allergy; GRP78, glucose regulated protein-78; HMGB-1, High mobility group Box 1; HSA, human serum albumin; IFN, Interferon; IIR: intestinal ischemia reperfusion; IgE, Immunoglobulin E; IL, Interleukin; LT (C4) (s), leukotriene (C4) (synthase); MC, mast cell; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein; MLN, mesenteric lymph nodes; MRGPRX2, MAS Related GPR Family Member X2; MSU, monosodium urate; N/A, not available; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3; ns, not significant; OVA, Ovalbumin; p, phosho; PCA: passive cutaneous anaphylaxis; PGD (s), prostaglandin D (synthase); PI3Ks, Phosphoinositide 3-kinase; PKC, protein kinase C; PLCγ1, phospholipase C, gamma 1; phospholipase Cγ (PLCγ); PPAR, proliferator-activated receptor; RAGE, receptor for advanced glycation endproducts; RESV, resveratrol; Sirt, sirtuin; smad, an acronym from the fusion of *Caenorhabditis elegans Sma* genes and the *Drosophila Mad*, Mothers against decapentaplegic; SPL: spleen; Syk, spleen tyrosine kinase; TGFβ1, Transforming growth factor beta 1; Th, T helper cells; TLR4, Toll Like Receptor 4; TNF-α, tumor necrosis factor, alpha; TNFR1, tumor necrosis factor receptor 1; Tregs, regulatory T cells; TSLP, thymic stromal lymphopoietin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; wk, week

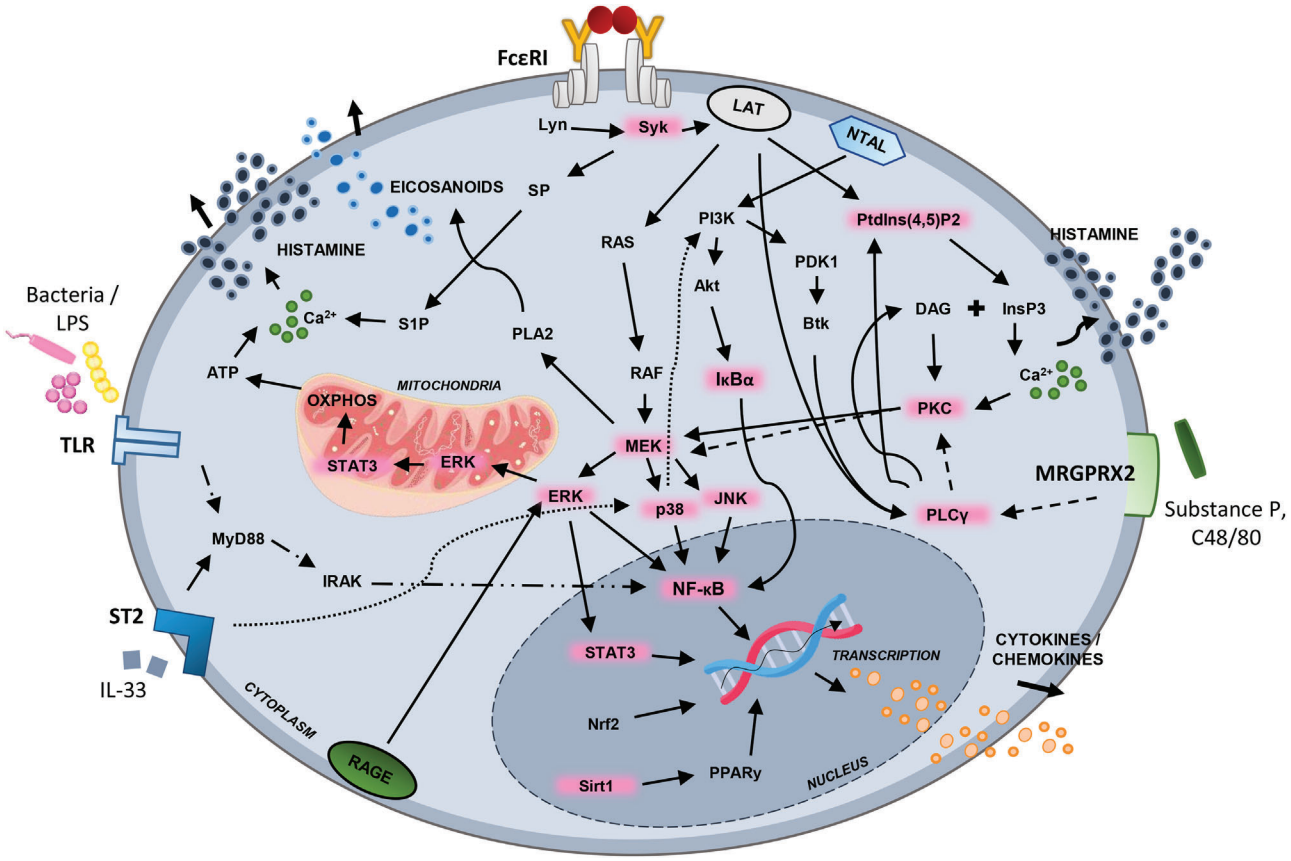

Figure 2. Signaling pathways in MCs affected by RESV. Activation of MCs is among others induced via receptors such as FcεRI, TLR, MRGPRX2, and ST2, leading to de novo synthesis of cytokines and chemokines as well as the release of prestored mediators like histamine. As stated in the text, RESV has been shown to affect various signaling molecules and transcription factors in cytoplasm, mitochondria as well as in nucleus involved in these signaling pathways (marked in pink).

RESV led to a decreased degranulation of mentioned mediators by about 80% (Table 1). We measured a reduction of IgE-mediated β-Hex release by about 75% of human intestinal mast cells (hiMC) in response to pretreatment with 50 µM RESV and a complete inhibition after treatment with 100 µM RESV.[35] The transcription factor signal transducer and activator of transcription (STAT) 3 was found to be present in mitochondria, and that mitochondrial STAT3 plays a major role in IgE-antigen-mediated mast cell exocytosis.[36] Moreover, ERK1/2 has been shown to phosphorylate STAT3 on the serine 727 residue. We could show that in IgE/antigen-activated hiMC 50 µM of RESV inhibited the phosphorylation of both nuclear and mitochondrial STAT3 and ERK1/2 by almost 100%. Thus, it can be concluded that RESV prevents activation of MCs also by inhibiting this pathway.[35] **Figure 2** summarizes signaling molecules and transcription factors in MCs that have been shown to be affected by RESV treatment.

Regarding degranulation, in vivo experiments show similar results (Table 2). In BALB/c mice, a commonly used strain in models of allergic diseases,[37] administration of 10 mg kg^{-1} RESV (10 mg kg^{-1} in 100 mL solution) resulted in a reduction of plasma histamine concentration, by about 50%, which was enhanced in sensitized mice challenged with 2,4-dinitrophenol (DNP)-human serum albumin (HSA).[38] The reduced histamine levels were concomitant with the reduced phosphorylation of the protein kinase C (PKC)-μ, spleen tyrosine kinase (Syk) as well as the phospholipase (PLC)-γ.[38] In an ovalbumin (OVA)-induced model of food allergy using BALB/c mice, RESV (20 mg kg^{-1} BW) decreased the serum histamine level by about 50%.[20] Furthermore, β-Hex levels were reduced to about 50% by RESV in the intestine of male Sprague–Dawley rats with intestinal ischemia-reperfusion (IIR).[39] C65BL/6 mice with pseudoallergy induced by C48/80 were pretreated with RESV (5, 10, 20 mg kg^{-1}) which resulted in a dose-dependent decrease of serum histamine levels (Table 2).[34] In addition, the number of degranulated MCs was reduced in a dose-dependent manner, too, whereas the maximum application concentration (20 mg kg^{-1}) led to a greater reduction (≈70%).[34]

3. RESV Attenuates the Synthesis of Arachidonic Acid Metabolites In vitro and In vivo

Newly synthesized lipid mediators are AAM such as prostaglandin D_2 (PGD_2) or leukotrienes (LTs), which are produced and released after MC activation by antigens. In mouse BMMC, which were sensitized with anti-DNP IgE and stimulated with DNP-bovine serum albumin (BSA), RESV reduced the release of LTs and PGD_2 at concentrations of both 100 and 10 µM (Table 2).[32] Moreover, the reduction of LTs was

about 99% after the application of 100 μM RESV, whereas PGD_2 was decreased by ca. 50% at 100 μM.[32] RESV at a concentration of 10 μM was used in a study by Li et al.[33] Here, LTC_4 and PGD_2 releases were reduced after the treatment of mouse BMMC with RESV by more than 70% (Table 1).[33] In in vivo experiments, lower doses of 5 mg kg^{-1} bodyweight (BW) as well as 10 mg kg^{-1} BW RESV led to a decrease in synthesis of PGDs and LTC_4s in OVA-sensitized BALB/c mice (Table 2).[40] Responsible for this result is probably the inhibition of the proinflammatory enzymes cyclooxygenase 2 (COX2) and 5-lipoxygenase (5-LO), which catalyze the generation of PGD_2 and LTC_4 out of arachidonic acid (AA), since the expression of these proinflammatory enzymes was reduced after RESV treatment, too.[40]

4. RESV Reduces the Expression of Cytokines and Chemokines In vitro and In vivo

Different from lipid mediators such as PGD_2 or LTC_4, which are synthesized in lipid bodies or nuclear/endoplasmic reticulum membranes and released through active transporters, de novo synthesized cytokines and chemokines packaged in secretory vesicles are released through constitutive exocytosis.[41] In RBL-2H3 cells, stimulated with either anti-DNP/DNP-HSA[42] or IL-33[43] the release of proinflammatory cytokines and chemokines, such as tumor necrosis factor α (TNF-α), IL-6, IL-4, IL-3 as well as monocyte chemoattractant protein 1 (MCP-1), was suppressed by RESV (Table 1).[42,43] This effect could be explained by the reduced phosphorylation of mitogen-activated protein kinases (MAPK) p38, extracellular-signal regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) which occurred following the treatment with RESV in a dose-dependent manner.[42] Besides MAPK, nuclear factor kappa B (NF-κB) pathway plays an important role in cytokine release from human MCs.[43,44] After incubation of IL-33 and IgE/antigen-stimulated RBL-2H3 cells with 10 μM RESV the phosphorylation of p38, inhibitor of NF-κB α (IκBα), and NF-κB subunit p65 was reduced by more than 50%.[43]

A reduced release of TNF-α, IL-13, and IL-6 by more than 40% was also detected in mouse BMMC stimulated with either IL-33 or anti-DNP-IgE/anti-IgE following the treatment with 25 μM of the polyphenol (Table 1).[7] Since it was found that the MAPK-activated protein kinase (MK)-2/3 mediated activation of phosphatidylinositol-3 kinase (PI3K)/Akt pathway is crucial for IL-33-induced IL-6 and IL-13 production in MCs[45] it can be suggested that RESV inhibits the synthesis of proinflammatory cytokines by targeting the MK2/3–PI3K/Akt axis.[7] Interestingly, the release of IL-6 and TNF-α was already diminished by about 60% in BMMC using a concentration of 10 μM RESV.[34] RESV treatment led to a decreased phosphorylation of protein tyrosine phosphatase 1B (PTP1B), which is suggested to be involved in FcεRI-dependent MC activation by regulating the Syk pathway.[33] Latter was deactivated, too, and it is known to be a central regulator of FcεRI signaling.[33]

Furthermore, Moon et al.[46] and Kang et al.[47] reported a reduction in mRNA expression of proinflammatory mediators like thymic stromal lymphopoietin (TSLP) by using human mast cell line (HMC)-1, which was stimulated with phorbol-12-myristate 13-acetate (PMA) and calcium ionophore A23187 after pretreatment with RESV. Higher doses of RESV (≥50 μM) resulted in a decreased release as well as mRNA expression of various cytokines and chemokines by about 60–70% (Table 1).[47] Following the treatment with 3 μM of RESV intracellular calcium levels were reduced resulting in a decreased production of receptor interacting protein (RIP) 2/caspase-1, which inhibited the activation of NF-κB or the phosphorylation of IκBα by about 50%.[46] The authors assumed that this effect was responsible for the reduced TSLP production by RESV.[46] As described above, the inhibition of NF-κB probably leads to an attenuation of allergic reactions by the decreased synthesis of other proinflammatory cytokines, too. Furthermore, Kang et al.[47] reported inhibitory effects on degradation of IκBα by RESV, which prevents nuclear translocation of p65 NF-κB. This could be, apart from the attenuated intracellular calcium levels, an explanation for the reduced expression of proinflammatory cytokines like IL-6 or TNF-α.[47]

In LAD2 the synthesis of MCP-1, TNF-α, and IL-1β was suppressed by RESV by about 50%.[34] In addition, we detected a dose-dependent decrease in mRNA expression of different chemokines, particularly C-X-C motif chemokine ligand (CXCL) 8, CC-chemokine ligand (CCL) 2, and CCL4, after the incubation of hiMC with RESV with a complete inhibition in response to 100 μM RESV.[35] As mentioned above, we found that RESV inhibited IgE mediated phosphorylation of STAT3 and ERK1/2, known to be involved in MC cytokine expression,[48] in hiMC by almost 100%, so we concluded that RESV prevents also the cytokine expression by inhibiting this pathway.[35] Aside from inhibition of crucial proinflammatory signal molecules, RESV leads to promotion of the mRNA expression of genes involved in the suppression of allergic reactions. RESV treatment of C48/80-stimulated LAD2 resulted in an increase of nuclear erythroid 2-related factor 2 (Nrf2) expression as well as heme oxygenase-1 (HO-1) and NADPH dehydrogenase quinone 1 (NQO1) generation, which are target genes of Nrf2.[34] Because the Nrf2/HO-1 pathway has been reported to play a role in IgE-dependent allergy, Nrf2 could act as a target for the therapy of MC-mediated allergic disorders.[34]

Various in vivo models reported a reduced expression of proinflammatory cytokines and chemokines in response to treatment with RESV (Table 2). In IL-33-stimulated male Sprague–Dawley rats treated with 5 mg kg^{-1} RESV plasma levels of IL-6 (≈50%), IL-13 (≈40%), TNF-α (≈60%), and MCP-1 (≈50%) were reduced.[43] Also, a decrease in serum levels of TNF-α (≈50%), IL-1β (≈40%), IL-18 (≈50%), and mRNA expression of IL-1β p17 (≈60%), and IL-18 (≈60%) was detected in rats suffering from IIR treated with 15 mg kg^{-1}.[39] Further cytokines such as IL-4 (≈40%), IL-5 (≈80%), or MCP-1 (≈50%) were less produced following the treatment with RESV in a models of passive cutaneous anaphylaxis (PCA) and eosinophilic rhinosinusitis with nasal polyps (CRSwNP) (Table 2).[40,38] Moreover, in female BALB/c mice with OVA-induced chronic allergic airway disease, transforming growth factor β1 (TGFβ1) expression in lung tissue was lowered by RESV (12.5 mg kg^{-1}).[49] Besides production of proinflammatory cytokines, the infiltration of chemokines, such as CXCL1, CXCL5, or CCL22, was inhibited by RESV in C65BL/6 gouty arthritis model (Table 2); however, the release of CXCL12 was promoted in joint tissue.[50] Thus, RESV increased the synthesis of sirtuin-1 (Sirt1) by 100% and the production of peroxisome proliferator-activated receptor (PPAR)-γ by about 60% in joints of gouty arthritis of C65BL/6

Table 3. Overview of the anti-inflammatory effects of RESV in randomized controlled trials.

Dosage RESV	Study type and duration	Study population	Results	Reference
100 µL per spray	RCT (1 month)	$N = 151$ adults with severe persistent AR Age: 18–60 years	Nasal symptoms ↓ Blood levels of IgE ↓ (≈40%), IL-4 ↓ (≈30%), and TNF-α ↓ (≈10%) Eosinophile number in blood ↓ (≈80%)	[53]
RESV + carboxymethyl-β-glucan (100 µL per spray)	RCT (2 months)	$N = 68$ children with AR Mean age: 7.9 years	Itching, sneezing, rhinorrhea, and obstruction ↓Antihistamine use ↓	[55]

AR, allergic rhinitis; IgE, Immunoglobin E; IL, Interleukin; RCT, double-blind randomized controlled trial; RESV, resveratrol; TNF-α, tumor necrosis factor alpha.

mice.[50] It is suggested that Sirt1 inhibits the infiltration of inflammatory cells as well as the secretion of proinflammatory molecules through its downstream molecule PPAR-γ.[50] Further, serum levels of IL-8 were suppressed by RESV in C65BL/6 mice with pseudoallergy (Table 2).[34] In an AD mouse model protein expression of TNF-α (≈60%), IL-1β (≈60%), and high-mobility-group protein (HMGB)-1 (≈70%) was reduced in skin, whereas the levels of IL-4 (≈70%) and interferon (IFN)-γ (≈50%) were decreased in the serum after the treatment with RESV (20 mg kg^{-1}) (Table 2).[51] Binding of the nonhistone chromatin-associated protein HMGB1 to receptor for advanced glycation end products (RAGE) activates a signaling pathway through ERK and NF-κB.[51,52] Since the HMGB1 signaling induces the generation of proinflammatory mediators the authors suggested that this pathway might be a potential therapeutic target in skin inflammation.[51]

5. RESV Attenuated Symptoms of Allergic Rhinitis in Randomized Controlled Trials

Not only in vitro and in vivo studies reported an attenuation of proinflammatory mediators and mechanisms. Beneficial effects of RESV on AR were additionally detected in randomized controlled trials (RCTs). AR is an IgE-mediated inflammatory disease of the upper respiratory tract, particularly of the nasal mucous membranes, which is caused by the interaction of allergens.[53] Diseases of the upper respiratory system are characterized by a common mechanism in the type 2 inflammatory pathway mediated by several inflammatory cells, such as eosinophils, mast cells, basophils, Th2 cells, or IgE-producing B cells, which release several mediators, chemokines, and cytokines.[53,54] In this context, MC mediators are released upon IgE-dependent mechanism in AR, but they can also induce IgE generation in B cells.[53] Once produced, local IgE acts on the FcϵRI receptors of tissue-resident MCs and basophils which results in the release of histamine or leukotrienes leading to edema, vasodilation, and bronchoconstriction.[54] Adult AR patients treated with RESV (100 µL per spray) showed a reduction in nasal symptoms compared to the placebo group. In this context, this polyphenol led to a decrease of IgE (≈40%), IL-4 (≈30%), TNF-α (≈10%), and eosinophil levels (≈80%) in the blood of the participants. Additionally, RESV treatment improved the quality of life of adults with AR (**Table 3**).[53] Furthermore, in children with pollen-induced AR, intranasal administered RESV (100 µL per spray) combined with carboxymethyl-b-glucan resulted in a significant reduction of nasal symptoms, including itching, sneezing, rhinorrhea and obstruction, and antihistaminic consumption (Table 3).[55] This indicates that RESV could be used as an adjuvant substance in AR to attenuate the symptoms in children and adults, not only because this polyphenol has been found to be safe and well-tolerated at up to 5 g per day.[56] However, it cannot yet be concluded that RESV can generally be used successfully in allergy patients. More studies are needed to prove the use of RESV as a potential substance in the treatment of allergic diseases.

6. Challenges in Using RESV as Anti-Inflammatory Nutraceutical

The effective RESV dosage found in vitro (micromolar range) can hardly be reached by oral administration in vivo due to its in vivo bioavailability, making it difficult to identify the concentration at which RESV should be administrated to human subjects.[57] Thus, although RESV was shown to be safe in vivo, arguably one of the biggest challenges regarding the use of RESV as a potential adjuvant substance in allergic diseases is its poor bioavailability. After oral administration, more than 70% of RESV is absorbed by the gastrointestinal tract.[57] It is rapidly metabolized by phase II enzymes in the intestine and liver leading to the accumulation of glucuronides and sulfate conjugates in plasma as well as urine, and hence the very low bioavailability of RESV.[27,57,58] Furthermore, 75% of the total consumed RESV is excreted, while the remaining amount of RESV is metabolized and the highest concentration of free RESV in the serum is 1.7–1.9%.[15] Since RESV has a limited dissolution rate in the aqueous environment, a small increase in solubility can enhance its bioavailability.[59] Topical administration of RESV has been shown to be more effective compared to oral application, as its oral intake results in quick metabolization and excretion.[27] RESV must be administrated orally at relatively high, i.e., mM, concentrations to achieve efficacy in cutaneous applications.[60]

In order to improve RESV's poor bioavailability, various methodological approaches have been developed, including several delivery systems such as RESV encapsulation in lipid nanocarriers or liposomes, emulsions, micelles, insertion into polymeric nanoparticles, solid dispersions, and nanocrystals.[59] Using 3T3-L1 fibroblasts, it was shown that trans-RESV encapsulated in lipid nanocarriers or liposomes increased cellular RESV content in cells, whereas RESV liposomes showed better biological activity due to its higher physical and chemical stability at room temperature.[59] Further, self-microemulsifying drug

delivery systems with UDP-glucuronosyltransferase excipients have been developed to increase oral bioavailability by inhibiting enzyme mediated intestinal metabolism.[61] In vivo, the inhibitory excipients containing self-microemulsifying increased oral RESV bioavailability compared to free RESV- and excipients without inhibitory activities.[59,61] Besides, oral bioavailability of trans-RESV from a grapevine-shoot extract (Vineatrol30) was increased in healthy subjects by enhancing its absorption via micellar solubilization compared with the native powder.[62] As mentioned before, encapsulation in nanoparticles is another strategy to improve oral bioavailability of RESV. In this regard, RESV-loaded galactosylated nanoparticles enhanced the oral bioavailability of RESV in Sprague–Dawley rats as well as the anti-inflammatory efficacy of RESV-loaded galactosylated nanoparticles in RAW 264.7 cells.[63] In another work with Sprague-Dawley rats, the oral bioavailability of an amorphous solid dispersion of trans-RESV was examined by a Eudragit E/HCl solid dispersion prepared by a spray drying process.[64] The absolute oral bioavailability of trans-RESV from Eudragit E/HCl solid dispersion (10/90) was estimated to be 40%.[64] In addition, nanocrystals are a promising approach to improve the oral bioavailability of RESV. Plasma concentration profile of trans-RESV nanocrystals has been shown to be enhanced compared to trans-RESV.[59] Overall, there are several strategies to increase the oral bioavailability of RESV.[59] However, the actual biologically effective concentration range of RESV in vivo needs to be determined in further studies.[57]

7. Conclusion

Based on the studies included in this overview, it can be concluded that RESV is able to attenuate proinflammatory, particularly IgE-dependent MC-mediated reactions in vitro and in vivo. Beneficial effects of this polyphenol were also reported in RCTs; thus, it can be assumed that RESV might be successful in alleviation of allergic symptoms, especially allergic rhinitis, in humans. However, the poor bioavailability of RESV is a big challenge for using RESV as nutraceutical in allergic diseases in general. There are several strategies to favorably influence the pharmacokinetics of RESV and further clinical trials are needed investigating the oral bioavailability of RESV. Nevertheless, the inhibition of the release of proinflammatory mediators including β-Hex, histamine, or cytokines/chemokines by MCs serves as an explanation for the anti-inflammatory impact of this polyphenol. Since RESV is a natural, safe, and well-tolerated substance it could be considered in future studies as a potential adjuvant or an alternative drug, especially when medication compliance is low because of adverse events caused by conventional therapy methods.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

All the authors have contributed equally to the writing and reviewing of this article.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Keywords

allergy, anti-inflammatory, mast cells, nutraceuticals, resveratrol

Received: March 14, 2022
Revised: April 29, 2022
Published online: June 3, 2022

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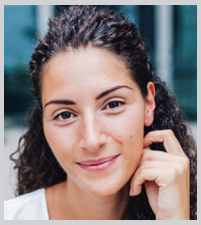
Mehtap Civelek, since May 2022: Postdoc at Section of Experimental Oncology and Nanomedicine (SEON), at University Hospital Erlangen, Germany; Postdoc at the Institute of Nutritional Medicine of University of Hohenheim, Germany; PhD, Institute of Nutritional Science of Justus Liebig University Giessen, Germany; study of nutritional medicine (Master of Science) at University of Hohenheim, Germany; study of nutritional sciences (Bachelor of Science) at Justus Liebig University Giessen, Germany.



Sabrina Bilotta Ph.D., candidate at the University of Hohenheim in Germany. She obtained her Master's degree in Agricultural Science in 2018. In 2019 she continued her research at the Institute of Nutritional Medicine in the field of mast cell biology. Focus is lead on the effects of secondary plant substances like resveratrol on mast cell activity and related signaling molecules and pathways in cellular and mitochondrial fractions of mast cells.

Axel Lorentz Associate Professor at the Institute of Nutritional Medicine, University of Hohenheim, and head of the mast cell research group. He studied biology, received his doctorate in genetics, was a postdoc in the fields of biochemistry and gastroenterology and habilitated in immunology. His focus is on mucosal immunology and in particular the role of mast cells. He is interested in the development of new therapeutic approaches, especially in mast cell-associated and intestinal diseases. The research aims to investigate the effects of natural bioactive compounds and the role of the circadian clock and microbiota in mucosal immunology.

DISCUSSION

MC play important roles in inflammatory reactions. Besides their function as main effector cells of IgE-mediated allergic reactions, they are also involved in gastrointestinal disorders like IBD. Polyphenols were previously shown to have a wide range of immunomodulatory effects which makes them a potential alternative and/or additive therapeutic agent in the treatment of OVA-induced allergic enteritis and IL-$10^{-/-}$ colitis which have encountered an increasing prevalence throughout the last decades. We therefore aimed to explore the effects of resveratrol on hiMC activity. Since degranulation is the most important activation signal in MC, we wanted to examine the inhibitory effect of this polyphenol on the degranulation related signaling cascade including the signaling molecules STAT3 and ERK1/2, which both contribute to mitochondrial dependent activation of the exocytosis process. Additionally, we checked on effects of resveratrol on MC associated diseases like FA induced enteritis and murine IL-$10^{-/-}$ colitis *in vivo*.

MC exert their inflammatory activity by the release of a variety of mediators, such as histamine, via degranulation processes after being stimulated by e.g. IgE. In IgE-dependent activated hiMC, we detected reduced degranulation in direct comparison to IgE-dependent stimulated but resveratrol-untreated controls. This inhibitory effect was detected with a concentration starting at 10 µM with significant dose-dependent inhibition and the strongest effects detected at a concentration of 100 µM. These observations, which are dependent on dose, were also made in mRNA expression levels of C-X-C motif chemokine ligand *(CXCL) 8,* C-C motif chemokine ligand *(CCL) 2, CCL3 and CCL4*. Dose-dependent responses of resveratrol were previously shown in other MC models [138-145] but interestingly, optimal dose concentration seems to be dependent of the respective MC models used, as implicated by the variability and amount of different study results [22].

Not only polyphenols such as resveratrol were found to display inhibitory effects on different MC models [138-145], but also experiments with the flavonoids nobiletin and tangeritin displayed inhibitory effects on IgE-dependent as well as IgE-independent hiMC activation [115]. Moreover, nobiletin resulted in reduced symptoms in murine IL-$10^{-/-}$ colitis and markers of fibrotic collagen deposition and expression in human intestinal fibroblasts [128]. Overall, nobiletin showed stronger effects than tangeritin [115]. Therefore, we wanted to compare the effects of resveratrol in direct comparison to that of the citrus flavonoid. Inhibition of degranulation and cytokine mRNA expression was significant for both substances. In direct comparison, significance for resveratrol was found to be stronger than that of nobiletin. The degranulation process of MC is dependent on several factors like Ca^{2+} levels [34], diverse signaling cascades [22] like the MAPK/ERK pathway and the presence of ATP generated from

OXPHOS in mitochondria [36]. Activity of STAT3 is inhibited by its specific inhibitor stattic [146] and we demonstrated that small natural components such as nobiletin and resveratrol show inhibitory effects comparable to stattic when focusing on mRNA expression of *CXCL8, CCL2, CCL3, CCL4* and tumor necrosis factor (*TNF-) α.*

With STAT3 playing such an important role in degranulation processes of MC [37], we additionally checked on effects of resveratrol as well as nobiletin on phosphorylation of STAT3 in MC after FcεRI-dependent activation. The study of Erlich and colleagues showed that phosphorylation of STAT3 on its serine residue 727 is driven by ERK1/2 [37], a signaling molecule shown to be involved in several cascades of MC biology [22]. As expected, the STAT3 inhibitor stattic was able to strongly reduce the activation of STAT3 in hiMC and further showed moderate inhibitory effects on phosphorylation of ERK1/2. In direct comparison to nobiletin, stattic showed stronger inhibition on ERK1/2 as well as on STAT3 activation. Resveratrol nearly abolished ERK1/2 activation by 100 % and showed similar effects like stattic on the inhibition of STAT3 activation, while nobiletin failed to significantly inhibit phosphorylation of STAT3. In the nucleus, STAT3 functions as a transcription factor [37, 147, 148]. Its activity drives chemokine and cytokine production, which are further released by MC afterwards. Increase of chemokine and cytokine presence is a common result in inflammatory processes like allergies [149]. Thereby, each of the released pro-inflammatory signals serves different functions during immune responses, explained in the following. We observed increased mRNA expression of *Ccl2*, *Ccl3* and *Ccl4* in mouse bone marrow derived MC (BMMC) [129] and hiMC [116] which act as regulatory factors in immune, endothelial and chemotaxis regulation [150]. In pathogenesis of IBD, TNF-α and CCL2 are released during early stages of the inflammation process and are needed for sustaining colitis [151]. CCL2 further recruits macrophages to sites of allergen exposure and it was shown that blocking of CCL2 signaling pathway prevents Th2 response in allergic asthma [152]. Besides macrophages, neutrophils and basophils are attracted to sites of inflammation by the presence of CXCL8 & TNF-α [153-155]. Both are directly induced via MAPK signaling pathway [156, 157]. Nonetheless, there are studies which do not report such findings [158, 159]. Effects of resveratrol on ERK1/2 are pronounced, but it is worth mentioning that they are not limited to it. This was confirmed by reduced phosphorylation of kinases Akt or JNK after IgE-dependent activation [116]. MAP kinase ERK1/2 directly interacts with STAT3 [37], which has an important role in OXPHOS [38]. Therefore, we wanted to examine if both, STAT3 and ERK1/2, are expressed in mitochondrial fractions of hiMC. Isolation of hiMC from tissue does not provide large cell numbers. After optimization of the isolation protocol due to low available cell numbers, we fortunately were able to examine mitochondrial fractions of mature hiMC. Additionally, we detected both, STAT3 as well as ERK1/2 phosphorylation and that resveratrol

diminished their activation after FcεRI crosslinking. After already being detected in mitochondrial fractions of other MC models [37, 160], we underline the presence of these signaling molecules in mitochondria of hiMC as well as that their occurrence suggests the importance in terms of MC activity.

Here, the inhibitory effect of a polyphenol on signaling molecules STAT3 and ERK1/2 in hiMC as well as their mitochondrial fractions is shown. Previous experiments demonstrate that two inhibitors based on curcumin, a polyphenol from turmeric origin, could reduce phosphorylation of STAT3-S727 in mitochondria of RBL-2H3 cells additionally to the reduction of degranulation and cytokine release in murine and human primary MC, but also on histamine release in acute anaphylaxis in mice [160]. A resveratrol-caffeic acid hybrid was shown to affect acetylation together with phosphorylation of STAT3 on its tyrosine residue T705 in human cancer cell lines [161]. As previously shown, nobiletin and tangeritin affect MAPK/ERK1/2 signaling pathway important for cytokine expression in hiMC, too [115]. The observed effects for nobiletin and tangeritin are similar as for resveratrol, but tangeritin showed smaller inhibition on ERK1/2 activation and *CXCL8, CCL2* and *CCL4* mRNA expression than nobiletin [115], which in turn showed smaller effects than resveratrol in our studies. Concerning MC treatment, these observations underline the potential of natural derived substances in the regulation and inhibition of MC biology and activity.

Moreover, especially for hiMC, inhibitory effects of cinnamon extract [162] and its active compound cinnamaldehyde [163] were found. Degranulation could be reduced to a level of 20 % and nearly total inhibition of *CCL2, CCL3, CCL4* and *TNF-α* mRNA expression [162] was observed, which again is comparable with results obtained for stilbene resveratrol [116]. In human mast cell line HMC-1, picetannol, a resveratrol metabolite, was also able to reduce gene expression of *TNF-α* and *CXCL8* [164]. *Tnf-α* was strongly diminished in BMMC, either activated via FcεRI-dependent or LPS activation in the present findings [129] which extends results from previous experiments [142, 165].

Natural derived components do not only show these effects *in vitro*, but, more importantantly, are also observed *in vivo*. The involvement of MC in gastrointestinal disorders is well described [11, 117-121, 166]. In order to explore the role of resveratrol on OVA-induced allergic enteritis in BALB/c mice as well as murine IL-10$^{-/-}$ colitis, animals were treated with a daily concentration of 50 mg/kg bodyweight (BW) resveratrol. Resveratrol was applied via drinking water and concentration was adjusted on a daily basis. Egg white protein (OVA) induced allergy is a common model to examine FA in mice [117, 167-169]. In these experiments, OVA-induced enteritis displayed as a mild form of FA. Animals, on which allergy was induced via egg white

protein per gavage showed a slight increase in stool softening after the 4th of an overall of 6 gavages. Severe diarrhea as well as weight loss, described as one of the major common consequences of food allergies [117, 168, 170], did not occur. The absence of both symptoms could be the result of the short treatment time of 28 days. More obvious symptomatology may be appeared with ongoing OVA challenges and an elongated experiment time [171-173]. The absence of clear symptoms has already been observed in other studies [174, 175] as well as the later onset of severe diarrhea with ongoing challenges with the respective allergen [173].

A more potent outcome of symptoms was observed during the experimental setting of the murine IL-10$^{-/-}$ colitis model. Due to the missing anti-inflammatory IL-10, animals develop a spontaneous form of chronic colitis [76]. With its anti-inflammatory role by suppressing T cell and macrophage functions and keeping up the maintenance of mucosal homeostasis it is an indispensable cytokine for intestinal immunity regulation [76, 176]. Resveratrol was able to delay onset of symptoms in animals receiving the polyphenol daily via drinking water. The onset of symptoms in the knockout mice appeared 14 days later than in animals not being applied with resveratrol. However, even though there has been a later onset of symptoms, the survival rate did not differ from the control group receiving no additive (60 %). In both experimental settings, we observed increased MC numbers in duodenum and colon sections. Increased MC numbers were previously described in a model of OVA FA by Brandt and colleagues and other studies examining FA [117, 125, 169]. Elevated MC numbers were also described in patients suffering from IBD [120, 121]. In both cases, resveratrol was able to inhibit the increase of MC in the respective tissues in our studies. Elevated presence of MC is accompanied with elevated levels of MC associated parameters such as MC proteases [125, 141, 168], MC mediators like histamine [123, 141, 177] or membrane receptors like IL-3 receptor α chain (IL-3rα). IL-3rα acts as binding site for IL-3, an important murine MC growth factor [178]. Expression of IL-3rα was enhanced in OVA enteritis, which could be the reason for the increase of MC numbers. Interestingly, resveratrol was able to nearly block the induced IL-3rα expression. Increase of MC numbers and infiltration with inflammatory cells are correlated with histological changes in the intestine [168, 177, 179, 180]. These were clearly detectable in IL-10$^{-/-}$ mice and displayed as epithelial damage, a reduced number of goblet cells and increase of immune cell infiltration which were significantly elevated in knockout mice. Resveratrol application resulted in diminished scores that resembled the levels of control wildtype mice. In contrast, only a slight increase of epithelial disruption was observed in OVA enteritis which was not affected by resveratrol.

However, OVA models exploring the anti-allergic and anti-inflammatory role of diverse natural components are auspicious. Amongst these substances were polysaccharides from Aloe vera

gel (100 mg/kg) [181], polyphenols from Arecae semen (0.2 % weight/volume (w/v)) [125], fisetin (3 mg/kg/day) [126], dihydromyrecitin (10 mg/kg) [124], Chinese sweet tea (1 % w/v) [123] or *Citrus tachibana* leaf extract with its components tangeritin and nobiletin [182]. OVA-induced physiological changes like histamine and MC protease 1 increase, as well as onset of diarrhea and increase of rectal temperature were diminished with resveratrol treatment for 13 days [141]. In IL-$10^{-/-}$ colitis, attenuation of colitis symptoms was achieved with cinnamon extract [127] and nobiletin [128] in a more distinct way than resveratrol did. Noteworthy, both, resveratrol and nobiletin delayed onset of specific colitis symptoms during the experimental course [128, 129]. It seems that some of these natural substances display a more auspicious effect than resveratrol in the treatment of gastrointestinal disorders [127, 128, 182]. These varying effects may be due to the different bioavailability properties of each substance, which is based, among other things, on the chemical structure of the substances. For nobiletin, in comparison to other polymethoxyflavones [183], bioavailability is reported to be better, but usually, overall bioavailability of these substances is stated to be extremely low [184-186]. A study of Singh showed that the maximum concentration of nobiletin in plasma of rats was about 1.8 µg/mL (corresponds to 4.47 µM) after uptake of a single oral dosage of 50 mg/kg [187] or about 9 µg/mL (22.37 µM) if nobiletin was dissolved in corn oil [188]. Same as for nobiletin [184-186], water solubility of cinnamon and cinnamon components is low [189]. Studies examining bioavailability of cinnamon extract are lacking. A study of Han & Cui showed that cinnamon oil was more efficient if carried in liquid-loadable tablets [189]. Zhao et al. were able to show that cinnamaldehyde, one of the major active components of cinnamon [163], had a half-life of 6.7 h in rats [190].

For resveratrol, bioavailability is reported to be low [132] due to its transformation into glucuronide and sulphate derivates in the intestine and the liver [134]. Orally ingested resveratrol is highly absorbed (about 70 %) [191, 192], and mainly excreted unmetabolized (about 75 %) [136]. The highest amount of free resveratrol was shown to be at levels of 1.7-1.9 % [136]. Plasma peak concentrations in humans are ranging from 71 ng/mL (0.31 µM) up to 634 ng/mL (2.78 µM) after a single dose of a 500 mg capsule and a tablet-juice mix containing 2125 mg resveratrol, respectively. After repeated doses of resveratrol (500 mg or 2000 mg capsule, respectively), peak plasma concentrations vary from 44 ng/mL (0.19 µM) up to 1274 ng/mL (5.58 µM) [191]. However, the review of Springer & Moco, 2019 [191] also shows that neither the dose administered nor the delivery form says anything about the amount of resveratrol that reaches the plasma and is thus available for other organ tissues. Plasma half-life of resveratrol was 9.2 h in humans [193] and 5.6-22 h (oral gavage of 312.5-1250 mg/kg, respectively) in male rats [194]. Half-life was shorter for mice than rats. In male mice, a half-life of 0.29-7.1 h was observed after oral gavage of 625 mg/kg and 2500 mg/kg trans-

resveratrol, respectively [194]. Part of future research must be to increase the bioavailability of resveratrol. Increase of uptake may be achieved by encapsulation with carrier substances [195, 196]. Although no optimal encapsulation strategy for resveratrol seems to have been developed so far, numerous studies indicate positive effects with regard to inflammation. Thereby, effects were observed either if resveratrol was applied via oral gavage [141, 197], via chow [167, 198] or as additive in drinking water [199, 200]. When comparing the different uptake routes, it becomes clear that there are also some strong differences in the administered concentrations of resveratrol. In *in vitro* studies, concentrations used for the experiments are ranging from very low (0.03 µM) [144] to high (200 µM) doses [145]. In human trials, doses used are ranging from 10 mg to 5 g [201-203]. Even if these concentrations were reported to be safe [204], it was previously shown that concentrations above 500 mg provoked mild to moderate gastrointestinal disorders [203, 205]. Capsules or tablets with a single dose of 500 mg are the highest commercially available single doses of resveratrol [206]. The in our study used concentration of 50 mg/kg BW in mice corresponds a dose of 243-324 mg in adult humans weighting 60-80 kg, respectively [207, 208]. These concentrations are in the range of the commercially available doses of 500 mg, a dose that was shown to provoke mild gastrointestinal symptoms in some cases [203, 205].

In summary, small natural derived substances like nobiletin and especially resveratrol showed strong inhibitory effects on degranulation and cytokine expression in hiMC. Release of mediators is a consequence of ERK1/2 and STAT3 phosphorylation which is inhibited by resveratrol treatment, too. These signaling molecules are present in mitochondrial fractions of hiMC, displaying important functions in the degranulation process after MC activation. Phosphorylation of ERK1/2 and STAT3 was also inhibited by resveratrol in mitochondrial fractions from hiMC. *In vivo*, we demonstrated that resveratrol prevented anaphylaxis in OVA-induced allergic enteritis as well as delayed onset of clinical symptoms in IL-10$^{-/-}$ colitis. Tissue inflammation associated with both disorders were dimished down to the levels of respective controls and it is worth mentioning that the increase of MC numbers was inhibited in both experimental set-ups. This makes resveratrol a potential plant-derived substance for the treatment and/or prevention of MC associated diseases (Figure 5).

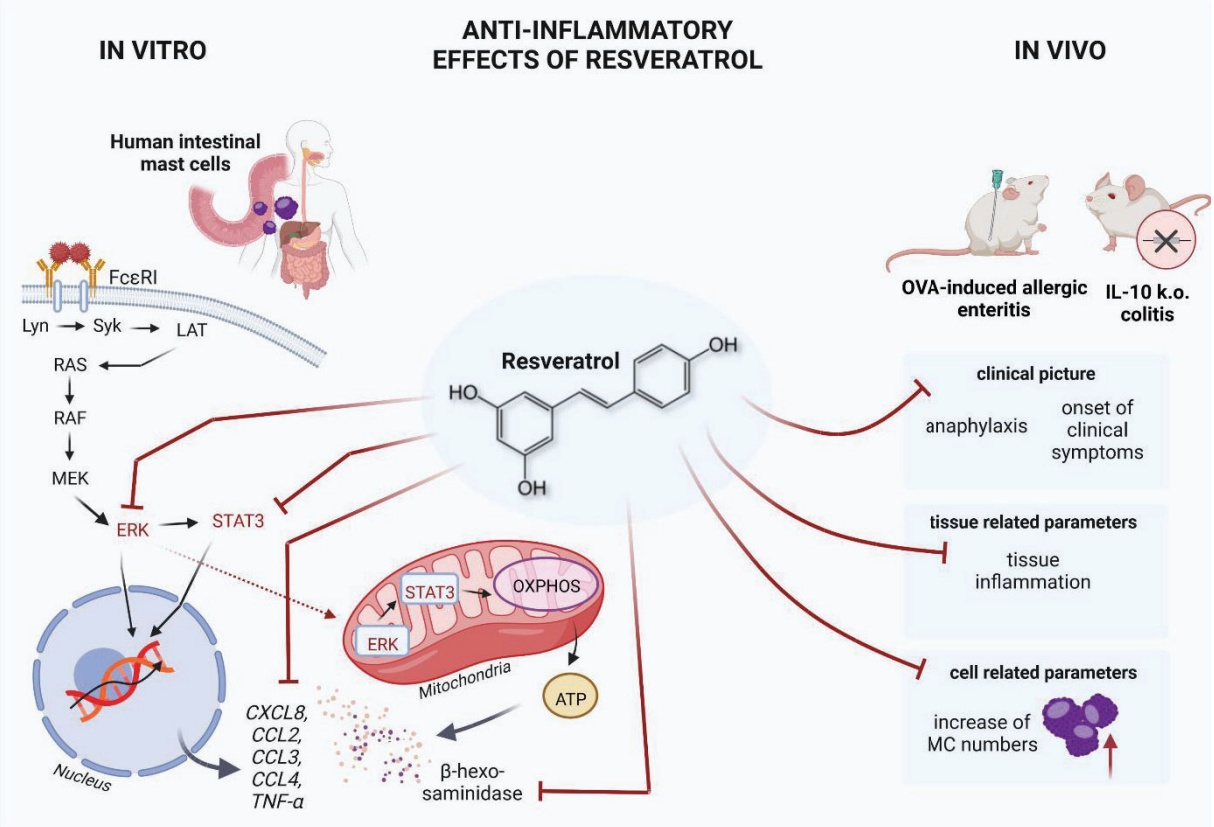


Figure 5 – Anti-inflammatory effects of resveratrol *in vitro* and *in vivo*. (created with BioRender.com)

As I mentioned at the beginning of this thesis, resveratrol could become a focus of attention in the future with regard to its use as a nutraceutical. Even though results of this thesis and many other studies examining the immunomodulatory effects of small natural derived components are promising, science as well as pharmacants and medicine producers face a major challenge here. Above all, the low bioavailability of natural substances such as polyphenols slows down their usage as nutraceuticals. Therefore, the focus should continue to be on improving bioavailability so that the substances may be used without restriction in the future and thus be used for the prevention but also improvement of various diseases. So far, there are many approaches to address the described problems.

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DECLARATION IN LIEU OF OATH

Declaration in lieu of an oath on independent work according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic
Immunomodulatory effects of resveratrol on human intestinal mast cell signalling in vitro and mast cell associated enteritis and colitis in mice
is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath. I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Göppingen, 04.11.2022 — S Bilotta

Place, date — Signature, Sabrina Bilotta

CURRICULUM VITAE

Personal data

Name	Sabrina Bilotta
Birthday, & -place	October 31, 1992 in Göppingen
Address	Dürerstraße 26/1, 73033 Göppingen
Phone	+ 49 152 38463907
E-Mail	sabrina.bilotta@uni-hohenheim.de

Working experience

November 2022 – ongoing

Regulatory Affairs Manager at Richard Wolf GmbH, Knittlingen

January 2022 - July 2022

Marketing employee at Karo Kauer Label GmbH, Eislingen/Fils

January 2022 – March 2022

Scientific assistant at the Institute of Nutritional Science & Prevention, University of Hohenheim

February 2019 – December 2021

Scientific staff at the Institute of Nutritional Science & Prevention, University of Hohenheim

Education

Since February 2019 – 2022

PhD student at the Institute of Nutritional Science & Prevention, University of Hohenheim

October 2015 – October 2018

M. Sc. Agricultural Sciences, University of Hohenheim

October 2012 – October 2015

B. Sc. Agricultural Biology, University of Hohenheim

September 2003 – June 2012

School education & Abitur, Erich-Kästner Gymnasium, Eislingen/Fils

Göppingen, 04.11.2022

Place, date

S. Bilotta

Signature, Sabrina Bilotta

ACKNOWLEDGEMENT

First of all, I would like to thank Prof. Dr. Axel Lorentz for his trust and for giving me the opportunity to do this work at the institute of nutritional medicine, even though I was not familiar with the subject at the time. Thank you for helpful advice, the always harmonious cooperation as well as your always open ear. I would also like to thank you for the lively exchange during my time at the institute. Keep up the team spirit!

I would also like to thank Prof. Dr. Stephan C. Bischoff, who, together with Axel, made this position possible for me. Further thanks go to my second examiner Prof. Dr. med. Dr. rer. nat. Sascha Venturelli, who with his expertise and research already dealt with the topic of resveratrol and agreed to evaluate this dissertation together with Prof. Dr. Axel Lorentz.

Furthermore, my thanks go to Prof. Ehud Razin and Dr. Bhargavi Lakshmi of the Hebrew University in Jerusalem, with whom we were able to implement the project funded by the German Israeli Foundation. It is a pity that the visit to Israel as well as the reception of the two in Germany was denied to us due to the Corona virus shortly before.

I would especially like to thank Yvonne Soltow, who was a tremendous support during my time at the Institute and who always gave me advice and support in all matters. It was very reassuring that even you were sometimes on the fence when it came to calculating. I would also like to thank you for your moral and energetic support during the animal experiment. For funny moments I would also like to thank my dear and bright Katja Petrov, who was unfortunately much too seldom on site.

I owe beautiful moments as well as a great and funny working atmosphere to our students Tobias Krohmer, Laura Weinhart and Johanna Schrainer. Johanna, I am glad to have found a very good friend in you. The same goes for Dr. Julia Zimmermann. Thank you for your bright and open nature and your always open ear.

In addition to Dr. Julia Zimmermann, I would also like to thank my other doctoral student colleagues Dr. Benjamin Seethaler, Lena Stiefvatter and Louisa Filipe Rosa for the much pleasure they gave me during my time at the institute. I would also like to thank Andreas Rings, for any technical questions and problems in the lab. I thank Nina Constroffer for many lunch breaks spent together, together with Yvonne and Julia, and the interesting and funny conversations. Thanks also to Dagmar Schuhmacher as part of the secretariat. I would like to thank all the staff members I had the pleasure to work with during my 3 years of employment at the Institute for the always warm and open working atmosphere.

I would like to thank Nadine Schart, Julian Arbogast and Maurice Frei for their support and cooperation in animal experiments, as well as their contribution to the present work in the context of their master and bachelor theses, respectively.

A great thank you also goes to the staff of the ZVH: without your friendly and open-minded nature, the work with the animals would not have been possible for me. This made my work with the mice, which I thought I could handle better, much easier. I thank you for the insanely great care of the animals. It is incredibly reassuring to know that the animals, even if they are laboratory animals, want for nothing.

The biggest thanks go to my mother Ute Bilotta and my boyfriend Julian Mack. Your constant encouragement helps me immensely in times of self-doubt. This also goes for my best friend Melissa Sajjadi, who is always there for me - HW is now a closed chapter. Special thanks also go to my friends Dr. Sarah-Lena Mack and Julia Ade, with whom I can share the PhD experience. Thank you all for your support - at all times!

DANKSAGUNG

Zuallererst möchte ich Dir, Prof. Dr. Axel Lorentz für dein Vertrauen, sowie für die Möglichkeit danken, diese Arbeit, auch als damals Fachgebietsfremde, am Institut durchführen zu können. Danke für hilfreiche Ratschläge, die stets harmonische Zusammenarbeit sowie Dein offenes Ohr. Ebenso möchte ich mich bei Dir für den regen Austausch während meiner Zeit am Institut danken. Behaltet euch diesen Zusammenhalt in der Arbeitsgruppe bei!

Dank gilt an dieser Stelle auch Prof. Dr. Stephan C. Bischoff, der mir als Institutsleiter, zusammen mit Axel, die Stelle ermöglicht hat. Weiterer Dank gilt meinem Zweitgutachter Prof. Dr. med. Dr. rer. nat. Sascha Venturelli, der sich mit seiner Expertise und Forschung bereits mit dem Thema Resveratrol beschäftigt hat und sich bereit erklärt hat, diese Dissertation zusammen mit Prof. Dr. Axel Lorentz zu bewerten.

Zudem gilt mein Dank Prof. Ehud Razin und Dr. Bhargavi Lakshmi der Hebrew University in Jerusalem, mit denen wir das von der *German Israeli Foundation* geförderte Projekt umsetzen konnten. Schade, dass uns der Besuch in Israel als auch der Empfang der beiden in Deutschland aufgrund des Corona-Virus kurz vor knapp verwehrt blieb.

Großer Dank gebührt vor allem Yvonne Soltow, die mir während meiner Tätigkeit am Institut eine wahnsinnige Unterstützung war und mir stets mit Rat & Tat in allen Belangen zur Seite stand. Es war sehr beruhigend, dass auch Du manchmal beim Rechnen auf dem Schlauch standest. Auch für deine moralische und tatreiche Unterstützung während des Tierversuchs möchte ich dir danken. Für lustige Momente möchte ich auch meiner lieben und aufgeweckten Katja Petrov danken, die leider viel zu selten vor Ort war.

Schöne Momente sowie eine tolle und witzige Arbeitsatmosphäre verdanke ich unseren Student*Innen Tobias Krohmer, Laura Weinhart und Johanna Schrainer. Johanna, ich bin froh in dir eine sehr gute Freundin gefunden zu haben. Das gilt ebenso für Dr. Julia Zimmermann. Danke für deine aufgeweckte und offene Art und dein immer offenes Ohr.

Neben Dr. Julia Zimmermann, danke auch meinen anderen Doktorandenkolleg*Innen: Dr. Benjamin Seethaler, Lena Stiefvatter und Louisa Filipe Rosa für viel Freude während meiner Zeit am Institut. Zudem danke ich Andreas Rings, für Rückfragen zu allen technischen Fragen und Problemen im Labor. Ich danke Nina Constroffer für viele gemeinsam verbrachte Mittagspausen, zusammen mit Yvonne und Julia, und die interessanten und lustigen Gespräche. Danke auch an Dagmar Schuhmacher als Teil des Sekretariats. Allen Mitarbeiter*Innen mit denen ich während meiner 3-jährigen Beschäftigung am Institut zusammenarbeiten durfte, danke ich für die stets herzliche und offene Arbeitsatmosphäre.

Nadine Schart, Julian Arbogast und Maurice Frei danke ich für Ihre Unterstützung und Ihre Mitarbeit im Tierversuch, sowie ihrem Beitrag zur vorliegenden Arbeit im Rahmen ihrer Master- bzw. Bachelorarbeiten.

Großer Dank gilt auch den Mitarbeitern der ZVH: ohne eure freundliche und aufgeschlossene Art wäre mir die Arbeit mit den Tieren so bestimmt nicht möglich gewesen. Das hat mir die Arbeit mit den Mäusen, von der ich dachte, dass ich sie besser wegstecke, ungemein erleichtert. Ich danke euch für die wahnsinnig tolle Versorgung der Tiere. Es ist ungemein beruhigend zu wissen, dass es den Tieren, auch wenn es sich um Versuchstiere handelt, an nichts fehlt.

Der größte Dank gilt meiner Mama Ute Bilotta und meinem Freund Julian Mack. Euer stetiger Zuspruch hilft mir ungemein, wenn ich wieder an mir selbst zweifele. Dies gilt auch meiner besten Freundin Melissa Sajjadi, die mir immer ein offenes Ohr ist – HW ist nun ein abgeschlossenes Kapitel. Besonderer Dank gilt auch meinen Freundinnen Dr. Sarah-Lena Mack und Julia Ade, mit denen ich die Erfahrung der Promotion teilen kann. DANKE euch allen für eure Unterstützung – zu jeder Zeit!