Knockout of the Bcmo1 gene results in an inflammatory response in female lung, which is suppressed by dietary beta-carotene

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Abstract Beta-carotene 15,15'-monooxygenase 1 knockout (Bcmo1−/−) mice accumulate beta-carotene (BC) similarly to humans, whereas wild-type (Bcmo1+/+) mice efficiently cleave BC. Bcmo1−/− mice are therefore suitable to investigate BC-induced alterations in gene expression in lung, assessed by microarray analysis. Bcmo1−/− mice receiving control diet had increased expression of inflammatory genes as compared to BC-supplemented Bcmo1−/− mice and Bcmo1+/+ mice that received either control or BC-supplemented diets. Differential gene expression in Bcmo1−/− mice was confirmed by real-time quantitative PCR. Histochemical analysis indeed showed an increase in inflammatory cells in lungs of control Bcmo1−/− mice. Supported by metabolite and gene-expression data, we hypothesize that the increased inflammatory response is due to an altered BC metabolism, resulting in an increased vitamin A requirement in Bcmo1−/− mice. This suggests that effects of BC may depend on inter-individual variations in BC-metabolizing enzymes, such as the frequently occurring human polymorphisms in BCMO1.

Keywords ATBC and CARET study · Beta-carotene 15,15'-monooxygenase 1 · Whole-mouse genome microarray gene expression · Inflammation · Vitamin A deficiency · ADH7 · LRAT

Introduction

Beta-carotene (BC) is an orange-colored dietary compound present in various fruits and vegetables. BC is regarded as an active antioxidant and is thus able to diminish radical-induced macromolecular damage [1]. Because of this, a high intake of BC might be expected to be beneficial, and indeed epidemiological studies have shown that a high dietary intake of BC is associated with a lower risk for cardiovascular diseases and several types of cancer, including lung cancer [2, 3]. Moreover, BC can be metabolized into vitamin A, thereby preventing vitamin A deficiency. Vitamin A deficiency can result in a wide variety of clinical manifestations, ranging from night blindness, growth abnormalities, an increased...
susceptibility for infections, as well as an increased mortality due to infectious diseases [4, 5]. In Western society, smokers are at risk for vitamin A deficiency in the lung [6, 7]. Since dietary BC intake is able to prevent vitamin A deficiency and is associated with a decreased lung cancer risk, it was hypothesized that BC supplementation in smokers would be beneficial and would decrease lung cancer incidence. In contrast to this expectation, BC supplementation alone (ATBC study, [8]) or in combination with vitamin E (CARET study, [9]) resulted in an increased lung cancer risk in smokers and asbestos-exposed subjects [8, 9]. An increased lung cancer risk was predominantly observed in smokers and asbestos-exposed subjects and not in former or non-smokers [10, 11]. Moreover, a 6-year follow-up of the CARET study revealed that females were more at risk for the development of lung cancer upon BC supplementation compared to male volunteers [12]. The underlying mechanisms are not precisely known, although several mechanisms have been suggested. Possibly, BC or BC metabolites can become pro-oxidants at high concentrations [13] or in combination with oxidative stress [14]. There is also evidence that BC supplementation can result in a changed regulation of the transcription factor retinoic acid receptor (RAR) [15]. However, the exact mechanism behind the possible adverse effect of a high BC intake is still poorly understood.

An important drawback in BC research is BC metabolism. First of all, BC metabolism in humans is highly variable [16, 17] and most of this variability can be attributed to differences in enzymatic activity of the beta-carotene 15,15'-monooxygenase 1 enzyme (BCMO1). The 267A>T and 379C>T polymorphisms in the BCMO1 gene are common with variant allele frequencies of 42 and 24%, respectively, in Caucasian population of the United Kingdom, and result in increased BC and decreased BC metabolite concentrations in plasma [18, 19]. Secondly, inter-species differences, especially in Bcmo1 activity, are the cause of the great differences in BC-metabolizing activity between humans and regularly used laboratory rodents. Rodents have a more active Bcmo1 enzyme variant than humans [20], and therefore BC supplementation to rodents results in a virtually complete conversion of BC and consequently in a high concentration of BC metabolites. Previously, a Bcmo1 knockout (Bcmo1<sup>−/−</sup>) mouse was established and characterized, and BC supplementation to these Bcmo1<sup>−/−</sup> mice resulted in increased BC plasma concentrations and BC accumulation in mouse lung tissue [21]. This Bcmo1<sup>−/−</sup> mouse is therefore a unique model to investigate the effects of BC itself.

The aim of our study was to investigate BC-induced gene-expression differences. Therefore, female Bcmo1<sup>+/+</sup> and Bcmo1<sup>−/−</sup> mice, unable to symmetrically cleave BC, were fed for 14 weeks with a vitamin A-sufficient diet (1,500 IU/kg) without (control) or with supplementation of BC (BC, 150 mg/kg diet) and microarray analysis was performed (whole-mouse genome arrays) on lung copy RNA (cRNA). We specifically chose female animals because the effects of BC were more pronounced and longer-lasting in female volunteers of the CARET study [12]. Moreover, wild-type (Bcmo1<sup>+/+</sup>) mice were analyzed to distinguish BC-induced effects from the effects due to the knockout of the Bcmo1 gene.

Materials and methods

Animals and treatment

Twelve female (Fe) B6129SF1 (Bcmo1<sup>+/+</sup>) and 12 female B6;129S-Bcmo1<sup>tm1dnp</sup> (Bcmo1<sup>−/−</sup>) mice, previously described by Hessel et al. [21] were used for the experiment. The mouse experiment was conducted in accordance with German animal protection laws by the guidelines of the local veterinary authorities. During the breeding and weaning periods of the mice, mothers were maintained on KLIBA 3430 chow containing 14,000 IU vitamin A/kg diet (Provia Kliba AG, Kaiseraugst, Switzerland). Five-week-old female Bcmo1<sup>+/+</sup> and Bcmo1<sup>−/−</sup> mice were caged in groups containing 2–4 siblings per group and were maintained under environmentally controlled conditions (temperature 24°C, 12 h/12 h light–dark cycle). Mice had ad libitum access to feed and water. Basic feed consisted of the palletized diet D12450B (Research Diets Inc, USA) with a fat content of 10%. The diet was modified to contain 1,500 IU vitamin A/kg of diet, which is a vitamin A-sufficient diet, and the control diet was supplemented with water-soluble vehicle beadlets (DSM Nutritional Products Ltd., Basel, Switzerland) containing dl-alpha-tocopherol and ascorbyl palmitate as stabilizers, as well as carriers such as gelatine, corn oil sucrose, and starch. The BC diet was supplemented with identical water-soluble beadlets containing 10% BC (DSM Nutritional Products Ltd., Basel, Switzerland) to generate 150 mg BC/kg diet. Beadlets were added by the manufacturer before low-temperature pelleting. Feed pellets were color-marked and stored at 4°C in the dark.

After 14 weeks of dietary intervention, six female Bcmo1<sup>+/+</sup> on the control diet (Bcmo1<sup>+/+</sup> Co), six female Bcmo1<sup>+/+</sup> on the BC diet (Bcmo1<sup>+/+</sup> BC), three female Bcmo1<sup>−/−</sup> on the control diet (Bcmo1<sup>−/−</sup> Co) and three female Bcmo1<sup>−/−</sup> on the BC diet (Bcmo1<sup>−/−</sup> BC) were randomly sacrificed during three subsequent mornings. Blood was collected from the vena cava after isoflurane and ketamine anesthesia. Blood was coagulated for at least
20 min at room temperature, cooled to 4°C, and then centrifuged. Lung tissue was removed, rinsed in phosphate buffered saline (PBS) and snap-frozen in liquid nitrogen. The lung tissues were stored at −80°C. Due to an insufficient number of female Bcmo1−/− mice in the original breeding pool, female Bcmo1−/− mice were used that were born 2 weeks later from an identical experiment, treated identically with three mice on the control diet and three mice on the BC diet to generate n = 6 per group, and sacrificed 2 weeks after the first group of mice.

HPLC separation of retinoids and carotenoids

Retinoids and carotenoids were extracted from lung tissues and plasma under dim red safety light (λ ≥ 600 nm). Briefly, tissues (20–40 mg) were homogenized in 200 μl of 2 M hydroxylamine (pH 6.8) and 200 μl of methanol with a glass homogenizer. For determination of BC and retinol in serum, 180 μl serum was added to 200 μl methanol. Then 400 μl acetone was added either to these plasma or tissue extracts. Extraction of carotenoids and retinoids was performed with petroleum ether. The extraction was repeated three times, and the collected organic phases were dried under nitrogen and redissolved in HPLC solvent (n-hexane/ethanol, 99.5/0.5). HPLC separation of carotenoids and retinoids and quantification of peak integrals was performed as previously described [22]. Solvents for HPLC and extraction were purchased in HPLC grade from Merck (Darmstadt, Germany).

Serum analysis of inflammation markers

Serum levels of the inflammatory cytokines; tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) were measured using the mouse serum adipokine Lincoplex Kit (Linco Research, Nuclilab, Ede, The Netherlands). Sera were diluted four times in HPE buffer (Sanquin, Amsterdam, The Netherlands) and measured according to the manufacturer’s protocol and as described before [23]. Cytokine levels were measured using the Bio-Plex system (Bio-Rad, Veenendaal, The Netherlands) and using BioPlex software (Bio-Rad). All individual samples were analyzed in duplicate and averaged. The detection limit of MCP-1 was 60 pg/ml of IL-6 was 5 pg/ml, and of TNF-α was 0.3 pg/ml.

Histology of the lung

Small pieces of upper-right-lung tissue of two randomly selected animals per group were fixed by immersion in 0.1 M sodium phosphate buffer containing 4% paraformaldehyde (pH 7.4) overnight at 4°C, thereafter dehydrated, cleared, and then paraffin-embedded. Three μm-thick sections were cut and stained with periodic acid schiff (PAS) and Mayer’s hematoxylin for histological analysis.

RNA isolation

Left-lung lobes were homogenized in liquid nitrogen using a cooled mortar and pestle. Total RNA was isolated using TRIzol reagent (Invitrogen, Breda, The Netherlands) followed by purification using RNeasy columns (Qiagen, Venlo, The Netherlands) using the instructions of the manufacturer. RNA concentration and purity were measured using the Nanodrop system (IsoGen Life Science, Maarsen, The Netherlands). Approximately 30 μg of total RNA was isolated with A_{260}/A_{280} ratios above 2 and A_{260}/A_{230} ratios above 1.9 for all samples, indicating good RNA purity. RNA degradation was checked on the Experion automated electrophoresis system (Bio-Rad, Veenendaal, The Netherlands) using Experion StdSense chips (Bio-Rad). Two samples did not meet RNA quality (Bcmo1+/+ mice, one on BC diet and one on control diet) and were omitted from the experiment.

Microarray hybridization procedure

The 4 × 44 k Agilent whole-mouse genome microarrays (G4122F, Agilent Technologies, Inc., Santa Clara, CA) were used. Preparation of the sample and the microarray hybridization were carried out according to the manufacturer’s protocol with a few exceptions as described previously [23, 24]. In brief, cDNA was synthesized from 1 μg lung RNA using the Agilent Low-RNA Input Fluorescent Linear Amplification Kit for each animal without addition of spikes. Thereafter, samples were split into two equal amounts, to synthesize Cyanine 3-CTP (Cy3) and Cyanine 5-CTP (Cy5) labeled cRNA using half the amounts per dye as indicated by the manufacturer (Agilent Technologies). Labeled cRNA was purified using RNeasy columns (Qiagen). Yield, A_{260}/A_{280} ratio and Cy3 or Cy5 activity were examined for every sample using the nanodrop. All samples met the criteria of a cRNA yield higher than 825 ng and a specific activity of at least 8.0 pmol Cy3 or Cy5. Then, 1,200 ng of every Cy3-labeled cRNA sample was pooled and used as a common reference pool. Individual 825-ng Cy5-labeled cRNA and 825-ng pooled Cy3-labeled cRNA were fragmented in 1× fragmentation and 1× blocking agent (Agilent Technologies) at 60°C for 30 min and thereafter mixed with GEx Hybridization Buffer HI-RPM (Agilent Technologies) and hybridized in a 1:1 ratio at 65°C for 17 h in the Agilent Microarray Hybridization Chamber rotating at 4 rpm. After hybridization, slides were washed according to the wash protocol with Stabilization and Drying solution (Agilent Technologies, Inc., Santa Clara, CA).
Technologies). Arrays were scanned with an Agilent scanner with 10 and 100% laser-power intensities (Agilent Technologies).

Data analyses of microarray results

Signal intensities for each spot were quantified using Feature Extraction 9.1 (Agilent Technologies). Median density values and background values of each spot were extracted for both the experimental samples (Cy5) and the reference samples (Cy3).

Quality control for every microarray was performed visually by using ‘Quality’ control graphs from ‘Feature’ extraction and M-A plots and boxplots, which were made using limmaGUI in R (Bioconductor) [25]. Data were imported into GeneMaths XT 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). Spots with a Cy5 and Cy3 signal twice above background were selected and log-transformed. The Cy5 signal was normalized against the Cy3 intensity as described before [26]. Supervised principal component analysis and heat mapping were performed using GeneMaths XT. Volcano plots were made using GraphPad Prism 5.0. Pathway analysis was performed using MetaCore (GeneGo), GO overrepresentation analysis (ErmineJ) [27] and literature mining.

Analysis of mRNA expression by real-time quantitative PCR (Q-PCR)

Real-time quantitative PCR (Q-PCR) was performed on individual samples (n = 6 per group) as previously described [28] to validate the microarray data. One microgram of RNA of all individual samples was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Q-PCR reactions were performed with iQ SYBR Green Supermix (Bio-Rad) using the MyIQ single-color real-time PCR detection system (Bio-Rad). Each reaction (25 μl) contained 12.5 μl iQ SYBR green supermix, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 8.5 μl RNase-free water and 2 μl diluted cDNA. The following cycles were performed: 1 × 3 min at 95°C, 40 amplification cycles (40 × 15 s 95°C, 45 s optimal annealing temperature, which was followed by 1 × 1 min 95°C and 1 × 1 min 65°C) and a melting curve was prepared (60 × 10 s 65°C with an increase of 0.5°C per 10 s). A negative control without cDNA template and a negative control without reverse transcriptase (–RT) were taken along with every assay. Data were normalized against the reference genes synthetin 5a (Stx5a) and ring finger protein 130 (Rnf130), which were chosen based on stable gene-expression levels between the mice on the microarray. Primers were designed using Beacon designer 7.00 (Premier Biosoft International, Palo Alto, CA). The following primer sequences were used: reference gene Stx5a forward 5'-TTAAAACACAGGAAACGATTCCAGG-3' and reverse 5'-CAGCGCAAGGAGACCAAACAAGA TG-3' (PCR at 60.0°C), reference gene Rnf130 forward 5'-ACAGGAAACCAGCCTGCTTG-3' and reverse 5'-ACCCGAAACACATCATTCTGGTTGATAGC-3' (PCR at 60.0°C), interferon, alpha-inducible protein 27 (Ifi27) forward 5'-GCTGCTACAGAGGACTACCC-3' and reverse 5'-TGCTGATTGGAGTGGCTACC-3' (PCR at 59.2°C), tripartite motif protein 12 (Trim12) forward 5'-GGAGAACCATCATGAGAAGCTCATAC-3' and reverse 5'-GGAAAGCCCTAGTGAGCTAGTGAG-3' (PCR at 58.3°C), ISG15 ubiquitin-like modifier (Isg15) forward 5'-TGAGAGCAATGGCCTGGGAC-3' and reverse 5'-GCGCTGCTGAAAGCCG-3' (PCR at 58.3°C), interferon-induced protein 27 (Ifi27) forward 5'-TGCGACTGGCATGAGCTAGTGAGG-3' and reverse 5'-CATGAAATGCCTCCAGTGAG-3' (PCR at 58.3°C). A standard curve for all genes, including reference genes, was generated using serial dilutions of a pooled sample (cDNA from all reactions of the groups analyzed). mRNA levels were determined using the ΔΔCT method (IQ5 software version 2.0; Bio-Rad). Analysis of individual samples was performed in duplicate.

Statistical analysis

Fold changes for both microarray gene expression and Q-PCR gene expression were calculated using mean log signal intensities. P values for differential expressions were calculated between two groups using two-tailed Student’s t test statistics on log intensity values. Changes were considered statistically different at p < 0.05. Effects of diet and genotype on concentrations of BC, retinol, and retinyl esters in lung and serum were analyzed using 2 × 2 factorial univariate ANOVA (SPSS version 15.0) and considered statistically significant when p < 0.05. Correlations and differences in correlations between BC concentration and BC metabolite concentrations in lung were tested (SPSS) and considered significant when p < 0.05.

Results

BC supplementation results in an increased BC concentration in lungs, particularly in Bc mol1−1− mice

BC supplementation resulted in elevated BC levels in serum and lung tissue of all mice (p < 0.05), but to a higher extent in Bc mol1−1− mice (Fig. 1a, b, p < 0.05). Retinol serum levels were not significantly different between the groups (Fig. 1c). Retinol and retinyl ester
concentrations in lung were increased in all mice upon BC supplementation \((p < 0.05)\), and as expected, to a higher extend in \(Bcmo1^{-/-}\) mice (Fig. 1d, e).

BC supplementation to \(Bcmo1^{-/-}\) mice restores differential gene expression to levels as observed in \(Bcmo1^{+/+}\) mice

To investigate the effects of BC on lung gene expression, microarray analysis was performed on cRNA of all mice. Of the 43,379 spots (number of spots on array minus control spots), 31,128 had a signal twice above background and were regarded as positive spots and included in the analysis. Supervised principal component analysis (SPCA) showed that animals could be discriminated according to their \(Bcmo1\) genotype and diet (Fig. 2a). BC supplementation resulted in 651 and 1,522 differentially expressed genes \((p < 0.05)\) in the \(Bcmo1^{+/+}\) and \(Bcmo1^{-/-}\) mice, respectively. Of these BC-regulated genes, 32 were commonly regulated in \(Bcmo1^{+/+}\) and \(Bcmo1^{-/-}\) mice and thus regulated independent of genotype.

We were interested in the effects resulting from BC accumulation in lung tissue. Since BC levels in lung tissue were higher in \(Bcmo1^{-/-}\) mice than in \(Bcmo1^{+/+}\) mice and, correspondingly, more genes were regulated in this group after BC supplementation, we further focused on effects of BC in \(Bcmo1^{-/-}\) mice. Of the 1,522 BC-regulated genes in the \(Bcmo1^{-/-}\) mice, 121 had an absolute fold-change (FC) >1.5-fold, with mainly down-regulated genes (Fig. 2b). We continued with these 121 BC-regulated genes and investigated whether these genes were regulated due to an effect of BC treatment in \(Bcmo1^{-/-}\) mice or due to an effect in the \(Bcmo1^{-/-}\) control mice. Therefore, we evaluated gene expression of these 121 genes in all four groups; \(Bcmo1^{+/+}\) control mice, \(Bcmo1^{+/+}\) mice with supplementation of BC, \(Bcmo1^{-/-}\) control mice and \(Bcmo1^{-/-}\) mice with BC supplementation. The absolute expression of each of the 121 genes was set on one for the \(Bcmo1^{+/+}\) control.
mice. Cluster analysis of the expression of these genes showed that the Bcmo1−/− control mice differed most in gene expression compared to the other three groups (Fig. 3a). The expression of these genes was compared to the expression in the Bcmo1−/− control mice and revealed that the average absolute fold-change was significantly different in the Bcmo1−/− control mice compared to the Bcmo1+/− control mice (p < 0.001), while there was no difference between the average expression of these 121 genes between the BC-supplemented Bcmo1−/− mice or Bcmo1+/− mice and the Bcmo1+/+ control mice (Fig. 3b).

Fig. 2 Supervised principal component analysis and volcano plot of the microarray data. a Supervised principal component analysis (SPCA) of the four groups; Bcmo1+/+ control mice (open circles), Bcmo1−/− BC mice (closed circles), Bcmo1−/− control mice (open squares), and Bcmo1−/− BC mice (closed squares). Bcmo1+/− mice are separated from Bcmo1−/− mice (x-axis) and mice on control diet are separated from mice on the BC diet (y-axis). b Volcano plot of the effect of BC supplementation on gene expression in Bcmo1−/− mice, showing the fold-change on the x-axis and the corresponding Student’s t test p value on the y-axis. In black are all the genes with a p value < 0.05 and an absolute fold-change > 1.5. Trim12 with a FC of 8.75 and p < 0.05 is outside the limits of this figure.

Fig. 3 Relative expression of genes regulated by BC in Bcmo1−/− mice in all groups. a Hierarchical clustering of all experimental groups based on the expression of all genes regulated by BC in the Bcmo1−/− mice with an absolute FC > 1.5 and p < 0.05. The expression of every gene is represented relative to the expression in Bcmo1+/+ control mice, which was set to 1.0. Genes and groups were clustered using Euclidean distance (linear scaled) UPGMA. b Graph representing the expression level of all genes regulated by BC in the Bcmo1−/− mice with an absolute FC > 1.5 and p < 0.05, in all groups. The expression of every gene is represented relative to the expression in Bcmo1+/+ control mice, which was set to 1.0. The absolute expression was significantly higher (p < 0.001) in Bcmo1−/− control mice compared to mice in the other groups. There was no significant difference in expression level between the other groups. ***p < 0.001
These data indicate that there was mainly an effect of the knockout of the *Bcmo1* gene in the mice fed the control diet, which was returned to normal levels as observed in the *Bcmo1*<sup>+/−</sup> mice by BC supplementation.

**Confirmation of array results by Q-PCR**

Real-time quantitative PCR (Q-PCR) was used to validate microarray results. Genes with the highest upregulation in *Bcmo1*<sup>−/−</sup> control mice compared to all other groups: interferon-induced protein 44 (*Ifi44*) (Fig. 4a), and interferon-induced protein 27 (*Ifi27*) (Fig. 4b); downregulation in *Bcmo1*<sup>−/−</sup> control mice: tripartite motif-containing 12 (*Trim12*) (Fig. 4c), and previously demonstrated retinoic acid responsiveness: ISG15 ubiquitin-like modifier (*Isg15*) [29] (Fig. 4d) were selected for quantification. Microarray results were confirmed by Q-PCR, resulting in a virtual identical and significant downregulation of *Ifi44*, *Ifi27*, *ISG15*, and an upregulation of *Trim12* in BC-supplemented *Bcmo1*<sup>−/−</sup> mice compared to *Bcmo1*<sup>−/−</sup> control mice in both microarray and Q-PCR analysis.

Gene expression and histology showed an increase in inflammatory responses in lungs of *Bcmo1*<sup>−/−</sup> control mice

We further classified the 121 genes that were regulated in *Bcmo1*<sup>−/−</sup> control mice with an absolute FC > 1.5 compared to BC-supplemented *Bcmo1*<sup>−/−</sup> mice into general biological process categories (Table 1). The main affected biological process category was the immune response and in particular direct interferon-induced gene response (Fig. 5). ErmineJ overrepresentation analysis and MetaCore analysis also showed that there was an overrepresentation of the inflammatory response in *Bcmo1*<sup>−/−</sup> control mice compared to BC-supplemented *Bcmo1*<sup>−/−</sup> mice using the whole dataset (ErmineJ; GO:0006954, Inflammatory response, *p* < 10<sup>−7</sup>, MetaCore; several maps belonging to the cell process immune response, *p* < 2 × 10<sup>−4</sup>).

Since most of the genes induced in *Bcmo1*<sup>−/−</sup> mice on the control diet were involved in inflammatory responses, we investigated whether the mice had an increased concentration of the inflammation-related cytokines: tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1). Serum concentrations of all three markers were below the detection limit (data not shown) while pulmonary infections usually would result in detectable serum concentrations of these markers [30].

We had additional material of two animals per group available for morphological analysis. Paraffin-embedded sections were stained with hematoxylin and PAS and then analyzed. Histology showed an overall healthy lung structure in all animals of the groups; *Bcmo1*<sup>+/+</sup> control mice, *Bcmo1*<sup>+/+</sup> receiving BC supplementation and *Bcmo1*<sup>−/−</sup> receiving BC supplementation (Fig. 6 a, b, e). The overall lung histology of the *Bcmo1*<sup>−/−</sup> control mice was however different from the other groups due to a general increase in the number of inflammatory cells in the lung and occasionally larger inflamed areas (Fig. 6d, e) resembling previously described lung histology upon vitamin A deficiency in rats [31].
Table 1 List of genes regulated by BC supplementation in female Bcmol^{-/-} mice with an absolute FC > 1.5 and p < 0.05

| Source name | Source symbol | Probe name | Systematic name | BC versus control | Bcmol^{1/-} versus BC | Control | BC |
|-------------|---------------|------------|-----------------|------------------|------------------------|---------|----|
| **Interferon-induced immune response** | | | | | | | |
| Adenosine deaminase, RNA-specific | Adar | A_51_P257134 NM_019655 | 0.05 | 0.07 | 1.60 0.01 | 1.81 0.01 | 1.13 0.04 |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 | Ddx58 | A_52_P385536 AK049305 | 1.03 0.88 | -1.50 0.04 | 1.93 0.01 | 1.24 0.24 |
| Eukaryotic translation initiation factor 2-alpha kinase 2 | Eif2ak2 | A_52_P559919 NM_011163 | 1.10 0.10 | -1.53 0.05 | 1.31 0.21 | -1.28 0.00 |
| GTase, very large interferon inducible 1 | Gvin1 | A_52_P535484 NM_029000 | -1.14 0.09 | -1.56 0.03 | 1.58 0.04 | 1.16 0.08 |
| Hect domain and RLD 5 | Herc5 | A_52_P679860 AK147960 | -1.04 0.36 | -1.67 0.03 | 1.81 0.03 | 1.13 0.02 |
| Interferon-activated gene 202B | Ift202b | A_51_P151182 NM_011940 | -1.14 0.45 | -2.39 0.04 | 7.08 0.00 | 3.38 0.00 |
| Interferon-activated gene 204 | Ift204 | A_51_P408346 NM_008329 | -1.08 0.49 | -1.90 0.04 | 2.67 0.01 | 1.52 0.00 |
| Interferon, alpha-inducible protein 27 | Ift27 | A_52_P90363 NM_029803 | 1.01 0.95 | -4.09 0.01 | 8.24 0.00 | 2.00 0.01 |
| Interferon, alpha-inducible protein 27 | Ift27 | A_52_P90364 NM_029803 | 1.06 0.71 | -3.57 0.01 | 7.04 0.00 | 1.87 0.02 |
| Interferon-induced protein 44 | Ift44 | A_52_P550858 AK085407 | 1.25 0.09 | -2.36 0.04 | 4.00 0.01 | 1.35 0.04 |
| Interferon-induced protein 44 | Ift44 | A_51_P487690 NM_133871 | 1.28 0.10 | -2.87 0.02 | 4.88 0.00 | 1.33 0.12 |
| Interferon-induced with helicase C domain 1 | Iftih1 | A_51_P387810 NM_027835 | 1.09 0.33 | -1.63 0.02 | 1.90 0.01 | 1.07 0.32 |
| Interferon-induced protein with tetratricopeptide repeats 1 | Iftil1 | A_51_P327751 NM_008331 | 1.12 0.50 | -2.87 0.03 | 4.36 0.01 | 1.36 0.09 |
| Interferon-induced protein with tetratricopeptide repeats 3 | Ifti3 | A_51_P350570 NM_010501 | 1.01 0.94 | -2.19 0.03 | 2.81 0.01 | 1.27 0.13 |
| Similar to interferon-induced protein with tetratricopeptide repeats 3 | Ifti3 | A_52_P663686 NM_00100585 | 1.04 0.78 | -2.46 0.03 | 3.19 0.02 | 1.25 0.14 |
| Interferon regulatory factor 7 | Irf7 | A_51_P421876 NM_016850 | 1.03 0.77 | -2.79 0.04 | 3.87 0.02 | 1.35 0.09 |
| ISG15 ubiquitin-like modifier | Isg15 | A_52_P463936 NM_015783 | 1.13 0.38 | -2.72 0.03 | 5.00 0.00 | 1.62 0.02 |
| Interferon dependent positive acting transcription factor 3 gamma | Isgf3 g | A_52_P127367 NM_008394 | 1.08 0.57 | -1.50 0.02 | 1.94 0.01 | 1.19 0.01 |
| Myxovirus (influenza virus) resistance 2 | Mx2 | A_51_P514085 NM_013606 | 1.07 0.54 | -2.49 0.03 | 2.82 0.02 | 1.06 0.70 |
| 2'-5' oligoadenylate synthetase 1A | Oas1a | A_52_P337357 NM_145211 | 1.04 0.70 | -2.16 0.03 | 3.37 0.00 | 1.50 0.02 |
| 2'-5' oligoadenylate synthetase 1B | Oas1b | A_52_P110877 NM_001083925 | 1.04 0.58 | -1.51 0.01 | 1.75 0.00 | 1.11 0.23 |
| 2'-5' oligoadenylate synthetase 1F | Oas1f | A_51_P154842 NM_145153 | 1.04 0.72 | -2.70 0.02 | 4.43 0.00 | 1.57 0.02 |
| 2'-5' oligoadenylate synthetase 2 | Oas2 | A_51_P277994 NM_145227 | 1.02 0.85 | -2.36 0.03 | 4.24 0.00 | 1.76 0.00 |
| 2'-5' oligoadenylate synthetase 3 | Oas3 | A_51_P472867 NM_145226 | -1.09 0.17 | -1.78 0.05 | 2.17 0.02 | 1.34 0.00 |
| 2'-5' oligoadenylate synthetase 3 | Oas3 | A_52_P516296 NM_145226 | -1.04 0.78 | -1.64 0.03 | 1.80 0.04 | 1.14 0.06 |
| 2'-5' oligoadenylate synthetase-like 1 | Oas1l | A_51_P437309 NM_145209 | 1.03 0.77 | -2.28 0.04 | 2.81 0.02 | 1.20 0.19 |
| 2'-5' oligoadenylate synthetase-like 2 | Oas1l | A_51_P387123 NM_011854 | 1.09 0.34 | -2.55 0.03 | 4.12 0.00 | 1.49 0.02 |
| Radical S-adenosyl methylion domain containing 2 | Rsad2 | A_51_P905132 NM_021384 | -1.03 0.80 | -2.18 0.01 | 3.51 0.00 | 1.65 0.03 |
| Radical S-adenosyl methylion domain containing 2 | Rsad2 | A_52_P670026 NM_021384 | -1.08 0.58 | -2.33 0.01 | 4.82 0.00 | 2.23 0.01 |
| Radical S-adenosyl methylion domain containing 2 | Rsad2 | A_51_P905134 NM_021384 | -1.12 0.29 | -2.03 0.02 | 3.33 0.00 | 1.84 0.01 |
| Receptor transporter protein 4 | Rtp4 | A_51_P304170 NM_023386 | 1.11 0.42 | -2.42 0.01 | 3.50 0.00 | 1.30 0.11 |
| Source name | Source symbol | Probe name | Systematic name | Bcmo1<sup>-/-</sup> Control | Bcmo1<sup>-/-</sup> versus Bcmo1<sup>-/-</sup> control | FC<sub>p</sub> | p | Bcmo1<sup>-/-</sup> versus control | FC<sub>p</sub> | p | Control BC versus Bcmo1<sup>-/-</sup> control | FC<sub>p</sub> | p | Control BC versus Bcmo1<sup>-/-</sup> versus Bcmo1<sup>-/-</sup> | FC<sub>p</sub> | p | Bcmo1<sup>-/-</sup> dependent lung inflammation 2047 |
|-------------|--------------|------------|----------------|-----------------------------|-----------------------------------------------------|--------|---|------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|--------------------------------|---------------------------|--------|---------------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|
| Source name                  | Probe name                        | Systematic name | FC   | p     | BC versus control | p     | FC   | p     | BC versus control | p     |
|-----------------------------|-----------------------------------|-----------------|------|-------|-------------------|-------|------|-------|-------------------|-------|
| Chemokine (C-X-C motif) ligand 3 | Ccl3                              |                  |      |       |                   |       |      |       |                   |       |
| Macrophage scavenger receptor 1 | Ccr1                              |                  |      |       |                   |       |      |       |                   |       |
| Pickl仲a factor               | Pickl仲a                         |                  |      |       |                   |       |      |       |                   |       |
| Tumor necrosis factor         | Tnf                               |                  |      |       |                   |       |      |       |                   |       |
| General immune response       | Slfn3                             |                  |      |       |                   |       |      |       |                   |       |
| Schla仲e 4                    | Slfn4                             |                  |      |       |                   |       |      |       |                   |       |
| Lactotransfase                | Ltf                               |                  |      |       |                   |       |      |       |                   |       |
| Immunoresponsive gene 1      | Irg1                              |                  |      |       |                   |       |      |       |                   |       |
| Placenta-specific protein 8  | Plac8                             |                  |      |       |                   |       |      |       |                   |       |
| Oxygen transport              | Eraf                              |                  |      |       |                   |       |      |       |                   |       |
| Cell-adhesion                 | Vnn1                              |                  |      |       |                   |       |      |       |                   |       |
| Lipid metabolism             | Apoc2                             |                  |      |       |                   |       |      |       |                   |       |
Table 1 continued

| Source name | Source symbol | Probe name | Systematic name | Bcmol<sup>+/−</sup> BC versus control | Bcmol<sup>−/−</sup> BC versus control | Control BC versus Bcmo1<sup>+/−</sup> | Bcmo1<sup>−/−</sup> BC versus Bcmo1<sup>+/−</sup> | FC | p  |
|-------------|---------------|------------|-----------------|-------------------------------------|-------------------------------------|----------------------------------------|----------------------------------------|-----|----|
| Serum amyloid A 1 | Saa1 | A_52_P318673 | NM_009117 | −1.02 0.71 | −1.73 0.02 | 1.84 0.02 | 1.08 0.25 | |
| Serum amyloid A 3 | Saa3 | A_51_P337308 | NM_011315 | 1.04 0.83 | −2.58 0.01 | 3.65 0.00 | 1.36 0.10 | |

**DNA or RNA modification**

| Source name | Source symbol | Probe name | Systematic name | Bcmol<sup>+/−</sup> BC versus control | Bcmol<sup>−/−</sup> BC versus control | Control BC versus Bcmo1<sup>+/−</sup> | Bcmo1<sup>−/−</sup> BC versus Bcmo1<sup>+/−</sup> | FC | p  |
|-------------|---------------|------------|-----------------|-------------------------------------|-------------------------------------|----------------------------------------|----------------------------------------|-----|----|
| Z-DNA-binding protein 1 | Zbp1 | A_51_P184936 | NM_021394 | −1.06 0.65 | −2.12 0.03 | 3.38 0.00 | 1.69 0.00 | |
| Poly (ADP-ribose) polymerase family, member 14 | Parp14 | A_51_P514712 | NM_001039530 | 1.00 0.96 | −1.53 0.04 | 1.77 0.02 | 1.15 0.03 | |
| DEXH (Asp-Glu-X-His) box polypeptide 58 | Dhhx58 | A_52_P223809 | NM_030150 | 1.06 0.46 | −1.98 0.03 | 2.73 0.01 | 1.30 0.04 | |
| Polymerase (DNA-directed), delta 3, accessory subunit | Pold3 | A_52_P271244 | NM_133692 | 1.01 0.79 | 1.78 0.01 | −1.49 0.05 | 1.18 0.12 | |
| Polymerase (DNA-directed), delta 3, accessory subunit | Pold3 | A_51_P490397 | NM_133692 | 1.20 0.13 | −1.68 0.00 | 1.58 0.00 | −1.28 0.10 | |

**Other**

| Source name | Source symbol | Probe name | Systematic name | Bcmol<sup>+/−</sup> BC versus control | Bcmol<sup>−/−</sup> BC versus control | Control BC versus Bcmo1<sup>+/−</sup> | Bcmo1<sup>−/−</sup> BC versus Bcmo1<sup>+/−</sup> | FC | p  |
|-------------|---------------|------------|-----------------|-------------------------------------|-------------------------------------|----------------------------------------|----------------------------------------|-----|----|
| D site albumin promoter-binding protein | Dbp | A_51_P184092 | NM_016974 | 1.29 0.60 | 1.76 0.01 | −2.99 0.01 | −2.19 0.05 | |
| Guanylate nucleotide-binding protein 3 | Gbp3 | A_51_P165244 | NM_018734 | 1.04 0.80 | −1.85 0.03 | 2.49 0.01 | 1.30 0.06 | |
| RAB6, member RAS oncogene family | Rab6 | A_51_P209873 | NM_024287 | 1.11 0.24 | 1.77 0.01 | −1.33 0.09 | 1.20 0.19 | |
| Thymidylate kinase family LPS-inducible member | Tyki | A_52_P188937 | NM_020557 | 1.07 0.54 | −1.77 0.03 | 1.86 0.03 | −1.01 0.90 | |
| Ring finger protein 213 | Rnf213 | A_51_P159503 | NM_001040005 | −1.00 1.00 | −1.69 0.04 | 2.18 0.01 | 1.29 0.02 | |
| Potassium channel tetramerisation domain containing 14 | Kctd14 | A_52_P491082 | NM_001010826 | −1.08 0.35 | 1.69 0.02 | −1.37 0.14 | 1.33 0.03 | |

**Unannotated**

| Source name | Source symbol | Probe name | Systematic name | Bcmol<sup>+/−</sup> BC versus control | Bcmol<sup>−/−</sup> BC versus control | Control BC versus Bcmo1<sup>+/−</sup> | Bcmo1<sup>−/−</sup> BC versus Bcmo1<sup>+/−</sup> | FC | p  |
|-------------|---------------|------------|-----------------|-------------------------------------|-------------------------------------|----------------------------------------|----------------------------------------|-----|----|
| NM_145449 | A_51_P184936 | NM_016974 | 1.05 0.48 | −1.64 0.01 | 2.04 0.00 | 1.18 0.07 | |
| NM_029733 | A_51_P165244 | NM_018734 | −1.10 0.27 | −1.51 0.00 | 1.65 0.00 | 1.20 0.03 | |
| NM_173743 | A_51_P500215 | NM_173743 | 1.01 0.94 | −2.49 0.02 | 3.89 0.00 | 1.55 0.02 | |
| XM_980696 | A_52_P286965 | XM_980696 | −1.18 0.08 | −2.01 0.04 | 1.99 0.06 | 1.17 0.06 | |
| NM_001081215 | A_51_P234274 | NM_001018215 | 1.11 0.14 | −2.05 0.02 | 2.55 0.01 | 1.12 0.22 | |
| AK050122 | A_52_P803082 | AK050122 | −1.06 0.61 | −3.71 0.02 | 5.76 0.01 | 1.65 0.02 | |
| AK083106 | A_52_P132102 | AK083106 | 1.04 0.79 | 1.59 0.01 | −1.45 0.03 | 1.05 0.65 | |
| NM_199015 | A_52_P380369 | NM_199015 | 1.08 0.26 | −2.04 0.03 | 2.60 0.01 | 1.18 0.08 | |
| AK010014 | A_51_P582332 | AK010014 | 1.05 0.74 | −4.01 0.01 | 8.26 0.00 | 1.96 0.01 | |
| AK007163 | A_52_P686392 | AK007163 | 1.19 0.31 | −1.64 0.05 | 1.36 0.25 | −1.44 0.03 | |
| XM_136331 | A_52_P999964 | XM_136331 | −1.04 0.49 | −1.50 0.05 | −1.13 0.54 | −1.64 0.00 | |
| XM_992714 | A_52_P1187949 | XM_992714 | 1.04 0.58 | 2.49 0.03 | −1.83 0.13 | 1.31 0.10 | |
| XR_001627 | A_52_P666442 | XR_001627 | −1.10 0.22 | −2.14 0.01 | 1.84 0.01 | −1.05 0.75 | |
| XM_001002167 | A_52_P214437 | XM_001002167 | 1.05 0.82 | −1.75 0.04 | 1.28 0.40 | −1.44 0.06 | |
| XM_983705 | A_52_P764477 | XM_983705 | −1.14 0.11 | −1.53 0.00 | 1.67 0.00 | 1.25 0.01 | |
Expression of genes involved in BC metabolism are shifted towards an increased retinyl ester storage in Bcmo1−/− mice

To further investigate whether a lack of BC or BC metabolites could explain the transcriptional and histological increased inflammatory response in the Bcmo1−/− control mice, we investigated changes in downstream BC metabolism. First, we evaluated correlations between BC, retinol and retinyl ester concentrations in mouse lung. There was a significant positive correlation between retinyl ester and BC concentrations in lung tissue of Bcmo1−/− mice ($R = 0.90$, $p < 0.001$) (Fig. 7a) with a much lower slope in the Bcmo1−/− mice ($R = 0.87$, $p < 0.001$). There was no significant correlation between retinol and BC concentrations in lung tissue of both Bcmo1−/− as well as in Bcmo1−/− mice ($R = 0.46$, $p = 0.13$ and $R = 0.52$, $p = 0.10$) (Fig. 7b).

Additionally, the expression of genes involved in downstream BC metabolism and retinoic acid catabolism in the lungs was analyzed. Four genes involved in BC metabolism were differentially expressed in Bcmo1−/− mice compared to the Bcmo1−/− control mice (Table 2) with lecithin-retinol acyltransferase (Lrat) and alcohol dehydrogenase 7 class IV (Adh7) having the highest fold-change. The expression of Lrat, involved in the esterification of retinol into retinyl esters to store retinol [32], was 2.6 and 3.2 times up-regulated in Bcmo1−/− mice on the control and BC-supplemented diet, respectively, as compared to the Bcmo1−/− control mice. Moreover, Adh7, the enzyme with the highest affinity and the highest conversion rate in the metabolism of retinol into retinal [33] was 2.7 times lower expressed in the Bcmo1−/− mice on a control diet as compared to the Bcmo1−/− control mice. BC supplementation of Bcmo1−/− mice partly restored this decreased Adh7 expression.

### Discussion

In this study we investigated transcriptional pathways that are regulated by BC in vivo in the lung to obtain insight into the possible mechanisms underlying previously found adverse effects of BC supplementation. For this purpose, we used a Bcmo1−/− mouse model that is known to accumulate BC in serum and lung, as well as Bcmo1−/− mice that have increased BC metabolite concentrations upon BC supplementation. Lung gene expression was explored using whole-genome microarrays and revealed that mainly genes involved in inflammation were up-regulated in Bcmo1−/− control mice. Similarly, histological analysis revealed an increased infiltration of inflammatory cells in Bcmo1−/− control mice. BC supplementation of
BC restores Bcmo1−/− dependent lung inflammation

**Fig. 5** Biological processes affected in Bcmo1−/− control mice. Graph representing the number of genes differentially expressed in Bcmo1−/− control mice versus Bcmo1−/− BC-supplemented mice. p < 0.05 and absolute fold change > 1.5 per biological processes category

**Fig. 6** Histological analysis of lung tissue. Representative sections (right upper lobe) of the lung of Bcmo1+/+ mice (a, b) and Bcmo1−/− mice (c–e) on a control diet (a, c, d) or a BC-enriched diet (b, e). The lungs of Bcmo1−/− mice receiving the control diet (c, d) had a different appearance compared to the lungs of the other groups, with an increase in inflammatory cells and occasionally some inflamed area’s (d). Magnification: 40×

Bcmo1−/− mice restored inflammatory gene expression and decreased the infiltration of inflammatory cells to levels as observed in Bcmo1+/+ control mice. An increased inflammatory response in the lungs has previously been observed upon vitamin A deficiency [34] and since BC supplementation could reverse the increase in
inflammatory cells and inflammation-related gene expression, we hypothesize that these effects are due to increased dietary vitamin A requirement in Bcmo1−/− mice compared to Bcmo1+/+ mice. An explanation for this increased vitamin A requirement could lie in the altered downstream BC and vitamin A metabolism in Bcmo1−/− mice, resulting in a less efficient conversion of vitamin A into the bioactive form of vitamin A; retinoic acid. BC supplementation was able to restore these requirements despite the knockout of Bcmo1.

We observed an increase in inflammatory cells in lung tissue and an increase in the expression of genes involved in inflammation in Bcmo1−/− control mice, which was restored by BC supplementation to levels observed in Bcmo1+/+ mice. An effect of BC itself on the inflammatory response has not been reported, whereas vitamin A deficiency has been reported to increase inflammation in rats [31, 34]. Moreover, vitamin A supplementation in vitamin A-deficient populations reduced the number and severity of infections and decreased the infection-induced mortality rate [5]. Since vitamin A levels were approximately similar in lungs of Bcmo1−/− mice fed with a control diet and Bcmo1+/+ mice, the increased inflammatory response likely involves the bioactive vitamin A metabolite retinoic acid. Retinoic acid is able to bind to the transcriptional active retinoic acid receptor (RAR) or to the retinoid X receptor (RXR), thereby modulating transcriptional responses. Indeed, effects on the inflammatory response have been shown to be regulated through the transcription factor retinoic acid receptor (RAR) [35, 36]. For example, Th1/Th2/Th17 balance, which is involved in adaptive immune response and important in the onset of autoimmune diseases, asthma, and allergic diseases, has been attributed to RAR activity. Interestingly, interferon-stimulated genes, which were predominant in our study, can also be RAR-regulated [37, 38]. Retinoic acid can be present in two isomers; all-trans retinoic acid (atRA) and 9-cis retinoic acid (9cRA), which have different transcriptional activities. Both isomers are able to bind to RAR, while only 9cRA can bind to RXR. Since effects on immunological function have mainly been attributed to RAR functioning, a shift in the balance of atRA and 9cRA is very unlikely to explain our results. We explain the increase in the inflammatory response in lungs of Bcmo1−/− mice by a more general reduction in available retinoic acid in Bcmo1−/− control mice due to altered gene expression of downstream BC-metabolizing enzymes. The gene expression of Alcohol dehydrogenase 7 (class IV Adh7), a key enzyme in the conversion of vitamin A into retinal and an important step before the formation of biologically active retinoic acid [39], was 2.7-fold down-regulated in Bcmo1−/− mice compared to the Bcmo1+/+ mice on the control diet. Indeed, Adh7 activity has previously been shown to be inversely associated with the degree of

![Graph A](image1.png)

**Fig. 7** Correlation of BC and BC metabolite concentrations in lung. Correlation of a BC concentration and retinyl ester concentration (Bcmo1+/+: R = 0.90, p < 0.001; Bcmo1−/−: R = 0.87, p < 0.001) and b BC concentration and retinol concentration (Bcmo1+/+: R = 0.46, p = 0.13; Bcmo1−/−: R = 0.52, p = 0.10) in lungs of Bcmo1+/+ mice (open circles) and Bcmo1−/− mice (closed circles).

| Systematic name | Source symbol | Bcmo1+/+ | Bcmo1−/− |
|-----------------|--------------|---------|---------|
| Aldh1a2         | 1a           | 1.03a   | 1.25b   |
| Aldh1a3         | 1a           | −1.25b  | −1.43c  |
| Aldh2           | 1a           | 1.05a   | −1.18b  |
| Lrat            | 1a           | 1.58b   | 2.58c   |
| Adh7            | 1a           | −1.14a  | −2.67b  |

Different letters indicate significant differences (Student’s t test, p < 0.05)
inflammation in the human gastric mucosa [40]. Additionally, an interaction between vitamin A concentration and Adh7 expression was previously reported: a vitamin A-deficient diet led to a decreased fetal survival in Adh7 knockout mice compared to wild-type mice, but not on a vitamin A-sufficient diet [33, 41]. The 2.6-fold increase in lecithin-retinol-O-acetyltransferase (Lrat) in Bcmo1−/− mice was also striking. Lrat is the main enzyme [32] for conversion of retinol to retinylesters, a form in which vitamin A can be stored, indicating that vitamin A is rather esterified and stored in Bcmo1−/− lungs. Taken together, these findings suggest that an increased inflammatory response in control Bcmo1−/− mice may be due to decreased retinoic acid production. BC supplementation prevents these effects in the Bcmo1−/− mice, possibly because BC can substitute for retinoic acid production in a pathway dependent on beta-carotene dioxygenase 2 (Bdo2), an enzyme with the ability to asymmetrically cleave BC, thereby generating \( \beta \)-apocarotenals and \( \beta \)-ionone [42], which can subsequently be further metabolized into retinoic acid [43].

Together, the altered gene expression of key enzymes for vitamin A metabolism may result in a changed dietary vitamin A requirement in Bcmo1−/− mice, which changes the vitamin A-sufficient control diet for Bcmo1+/+ mice, into a mildly vitamin A-deficient diet for the Bcmo1−/− mice.

We demonstrated an altered BC metabolism in the Bcmo1−/− mice, which resulted, as intended, in an increased BC accumulation in lung and serum of the Bcmo1−/− mice. We also observed some differences in the gene expression of downstream BC-metabolizing enzymes. This changed metabolism might be important in understanding and investigating the physiological effects of BC in lung functioning, especially since there are large inter-human variations in BCMO1 activity due to polymorphisms in this gene. There were some interesting, unexpected changes in BC metabolite levels upon BC supplementation in Bcmo1−/− mice. First of all, there was an increase in retinol in lung upon BC supplementation in Bcmo1−/− mice. This increase in retinol is unlikely to be originating from metabolized BC. As far we know, only Bdo2 can serve as a partial alternative to Bcmo1 in BC metabolism. Bdo2 asymmetrically cleaves BC, thereby generating an increase in apocarotenals and ultimately into retinoic acid, which can be degraded by members of the Cytochrome P450 (CYP) family and in particular CYP26 [44, 45]. Retinol is probably not an intermediate in this alternative BC metabolism pathway. An increased retinol absorption in the lung, in combination with the (non-significant) decrease in retinol in plasma could theoretically clarify this observation. However, stimulated by retinoic acid 6 (Stra6), which is identified as a receptor for the blood retinol carrier; retinol-binding protein (Rbp) [46], was not significantly differentially expressed on the microarrays between the BC-supplemented and control diet-fed mice. A changed RBP-retinol complex has been associated with an increased delivery of retinol to different tissues [46, 47] and might possibly explain our retinol plasma and lung tissue concentrations. A second never reported interesting observation herein regarding BC metabolites in lung tissue was the tighter correlation of BC with retinyl esters than with retinol in lung tissue of Bcmo1+/+ mice. This implies that BC accumulation tightly regulates the retinyl ester pool. Lung tissue has relatively high retinyl ester pools [48]. The main role for retinyl esters in the lung is to ensure direct retinol delivery when there is an increased retinol demand in the lung [49], especially during fetal development. We found a significant 6.5-fold decrease in slope in the correlation of lung BC with retinyl esters in Bcmo1−/− mice compared to the Bcmo1+/+ mice, which implies decreased conversion efficiency in the Bcmo1−/− mice. This decreased efficiency was also found in people containing two snp’s in the BCMO1 gene, resulting in a decreased BCMO1 activity and a four-fold decreased conversion efficiency of BC as measured as the triglyceride-rich lipoprotein retinyl palmitate:BC ratio [18]. The surprising findings in BC metabolites in Bcmo1+/+ and Bcmo1−/− mice and the understanding of these differences are especially important in understanding the effects of BC in humans, in particular since downstream BC metabolism is different between mice and humans, and also between humans.

Since we find inflammatory gene expression to be increased, the question may be raised whether there was an unintended infection present solely in the Bcmo1−/− control mice. There are some reasons to believe that this was not the case. First, we examined plasma levels of the cytokines; MCP-1, TNF-\( \alpha \) and IL-6, which are highly increased and detectable upon infection [50]. These levels were below the limit of detection, which is representative for an uninfected state. In addition, the Bcmo1−/− mice both on a control diet and on a BC-supplemented diet were divided over two independent experiments. The significant up-regulation of the inflammatory response in the Bcmo1−/− control mice was similar in both experimental periods, making infection an unlikely explanation. Moreover, an increased inflammatory response in the lungs is often seen during vitamin A deficiency. An altered downstream BC metabolism in Bcmo1−/− mice, and thereby a higher vitamin A requirement in Bcmo1−/− mice is therefore a likely explanation.

We initiated this study to identify the possible pathways explaining possible harmful effects of BC. Therefore we used Bcmo1−/− mice, which are able to accumulate BC similar to humans. In this study, BC supplementation was beneficial in Bcmo1−/− mice, since BC supplementation reduced the inflammatory response in these mice and
generated a gene-expression pattern more similar to Bcmo1+/+ mice, as has been shown by cluster analysis. Although BC was beneficial in this model, these data also demonstrate that BC was able to influence the inflammatory response in the lungs. The harmful effects of BC have only been reported in smokers and asbestos-exposed subjects (ATBC, CARET). An important difference between smokers and asbestos-exposed subjects on one hand and non-smokers on the other, is that smoking causes an inflammatory response in the lungs, which is shown to be involved in the onset and progression of carcinogenesis [51–54]. Since we find differences in downstream BC-metabolizing enzymes, possible differences in these enzymes in smokers might give a clue in understanding the adverse effects of BC in smokers. Microarray analysis of small airway epithelium of smokers has revealed that several downstream BC metabolizing enzymes are indeed differentially expressed. Aldehyde dehydrogenase family 1, subfamily A3 (ALDH1A3) was five-fold up-regulated and ADH7 was six-fold up-regulated in smokers compared to non-smokers [55]. This paper implies that a proper retinoic acid synthesis is indispensable for correct functioning of the immune system. It would be of interest to investigate whether polymorphisms in BC-metabolizing enzymes in subjects of the ATBC and CARET study alter the lung cancer risk in smokers upon BC supplementation. Future subjects of the ATBC and CARET study alter the lung size that this was due to an increased vitamin A requirement in Bcmo1−/− mice as well as Bcmo1+/+ mice in combination with BC and smoke or inflammation induction are necessary to further investigate and explain exact mechanisms. Since the Bcmo1−/− mice seem to have a higher dietary need for vitamin A, this concentration should be increased to avoid effects of vitamin A deficiency.

All together, our results demonstrate that Bcmo1−/− mice on a control diet had an increased inflammation in their lungs, which was decreased to levels as observed in Bcmo1+/+ mice upon BC supplementation. We hypothesize that this was due to an increased vitamin A requirement in Bcmo1−/− due to impaired retinoic acid synthesis. Since the Bcmo1−/− control mice were mildly vitamin A-deficient, this study was unable to elucidate BC accumulation-regulated gene expression. Nevertheless, these results might be important for the human population since individuals with polymorphisms in the BCMO1 gene, with a high population frequency, may also have a higher risk for vitamin A deficiency, which is still a relatively frequently occurring state in developing countries as well as in certain groups of the Western society.

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