Title

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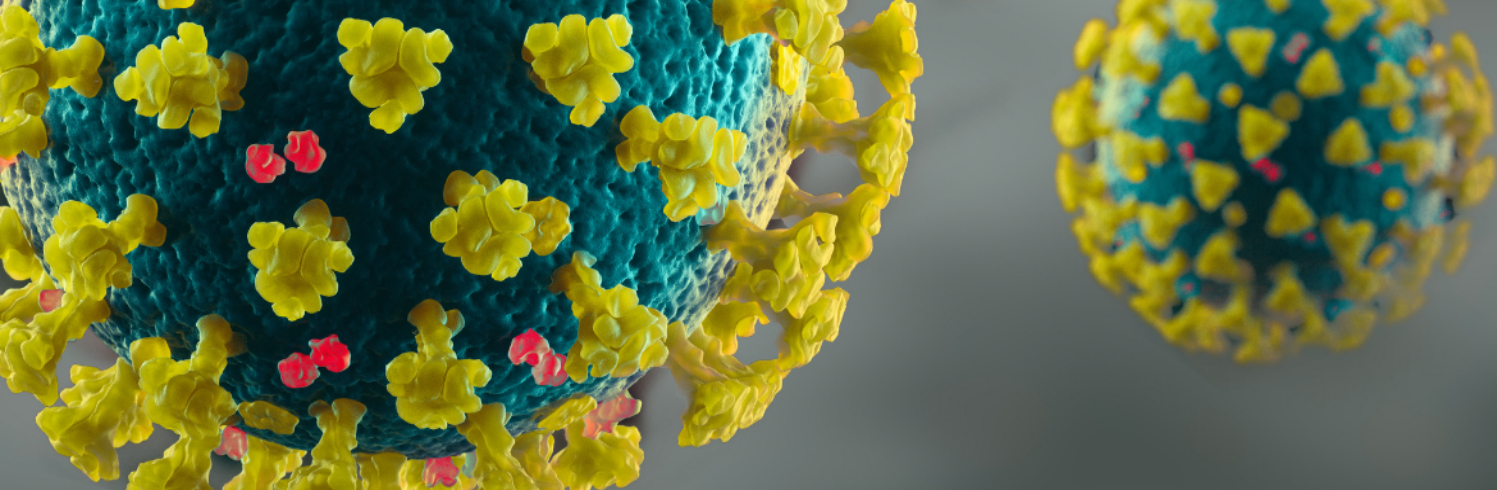
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Vitamin A metabolism and mucosal immune function are distinct between BALB/c and C57BL/6 mice

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The vitamin A metabolite retinoic acid (RA) has been reported to suppress Th1 responses and enhance Th2 responses. Here, we investigated whether differences in vitamin A metabolism could underlie the differences between C57BL/6 and BALB/c mice, which are reportedly seen as Th1 and Th2 responders, respectively. BALB/c mice were shown to have higher intestinal epithelial expression of RALDH1 (where RALDH is retinaldehyde dehydrogenase), and, consequently, higher RALDH activity in MLN-DCs, leading to an increased ability to induce IgA class switching in B cells. Furthermore, within BALB/c mice, induction of IgA secretion as well as increased accumulation of regulatory T cells (Treg) in the intestinal lamina propria was observed. Additionally, as BALB/c mice are more resistant to dextran sulphate sodium (DSS) induced colitis, mice that lacked vitamin A in their diet had a more severe form of DSS-induced colitis compared to control mice. Therefore, the level of RA production and consequently the degree of RA-mediated signaling is crucial for the efficiency of the mucosal immune system.

Keywords: BALB/c · C57BL/6 · FoxP3 · IgA · Intestine · Retinaldehyde dehydrogenase · Vitamin A

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Introduction

Vitamin A has long been known for its role in immunity, especially since vitamin A deficiency ablates proper mucosal immune responses, leading to diarrhea, infections, and early childhood mortality [1–3]. Vitamin A is a fat-soluble vitamin and is absorbed from the gastrointestinal tract. It can be absorbed in the form of preformed retinyl esters, as found in animal source foods such as liver, egg, fish, and whole fat dairy products [3]. Dietary vitamin A can also be obtained from provitamin A carotenoids as found in vegetables and fruits. Vitamin A is metabolized into the active metabolite retinoic acid (RA) in two oxidative steps. Vitamin A is first reversibly oxidized by alcohol dehydrogenases (ADH) to form retinaldehyde. Next, retinaldehyde is irreversibly metabolized to

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RA by one of the three members of the aldehyde dehydrogenase gene family, RALDH1, RALDH2, and RALDH3 (where RALDH is retinaldehyde dehydrogenase) [4–6]. The active metabolite RA binds to retinoic acid receptors (Rars) as well as retinoic X receptors, which in turn act as transcription factors that bind RA responsive elements within the promoter regions of target genes [7–9].

Expression of the genes encoding RALDH (*aldh1a1–3*) is associated with the mucosal immune system and the RALDH1 enzyme is reported to be functionally active within intestinal epithelial cells [10–12]. Also, DCs in Peyer's patches, MLNs, and intestinal lamina propria express RALDH enzymes, while splenic or peripheral LN DCs display only very low expression levels [13–17]. We have shown that RA itself directly regulates the level of *aldh1a2* (gene of RALDH-2) expression in BM-DCs as well as LN stromal cells in vitro, and that *aldh1a2* expression within the mucosal immune system requires dietary intake of vitamin A [18]. RA is reported to skew immunoglobulin class switching in B cells to an increased secretory IgA production [19–21], which protects the host against the vast array of microbes that are constantly present within the intestinal lumen. Moreover, generation of RA is crucial for the induction of gut-homing molecules $\alpha_4\beta_7$ and CCR9 on activated lymphocytes [15]. The induction of FoxP3 regulatory T (Treg) cells additionally relies on RA, in combination with TGF-β [14, 22–27]. Therefore, RA appears to be a key molecule that controls mucosal immune responses.

The genetic background of inbred mice was found to be a determining factor in the preferential induction of either a Th1 or Th2 immune response [28, 29]. The subset of T cells termed Th1 cells, which produce interleukin-2 (IL-2), interferon-γ (IFN-γ), and lymphotoxin, are important mostly for immunity against intracellular pathogens, such as *Leishmania major*. In contrast, Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which contributes to the direct enhancement of humoral-mediated immunity. T cells from C57BL/6 mice preferentially produce Th1 cytokines with high IFN-γ and low IL-4, whereas T cells from BALB/c mice favor Th2 cytokine production with low IFN-γ and high IL-4 when stimulated in vitro [28]. Interestingly, it has been shown that RA can suppress Th1 responses and enhance Th2 responses [15]. In this study, we therefore investigated whether C57BL/6 and BALB/c mice differ in the production of RA and in mucosal immune functions that are RA-dependent. We demonstrated that BALB/c mice showed higher expression levels of *aldh1a* genes and RA-mediated signaling within the small intestines (SIs) compared to C57BL/6 mice. MLN-DCs from BALB/c mice displayed higher RALDH activity, enabling these cells to induce more B cells to produce IgA in vitro, as well as more secretory IgA in vivo. Additionally, we found enhanced accumulation of B and T lymphocytes and higher percentages of FoxP3^{+} Treg cells in the SI. To show functional differences in the mucosal immune system between C57BL/6 and BALB/c mice, we confirmed earlier reports that BALB/c mice are more resistant to developing colitis [30–35] and showed that the availability of RA protected against dextran sulphate sodium (DSS) induced colitis in C57BL/6 mice. In conclusion, enhanced RA production in BALB/c mice is associated with the development of a more efficient mucosal immune system, while additionally the absence of vitamin A is associated with a more severe DSS-induced colitis in C57BL/6 mice.

Results

BALB/c express higher levels of *aldh1a* in the intestines compared to C57BL/6

Since BALB/c mice are known as prototypical Th2-type mice and RA has been described to skew T cells toward the Th2-type profile upon activation, while inhibiting Th1 cytokine production [15, 22], we hypothesized that the expression of RA-producing enzymes may be differentially expressed by BALB/c and C57BL/6 mice. Since the intestines are a site known to express RALDH enzymes important for immune homeostasis [10–12], we decided to compare *aldh1a* mRNA levels in the SIs and colon of both mouse strains with real-time PCR. Indeed, expression of *aldh1a1*, *aldh1a2*, and *aldh1a3* was significantly enhanced in BALB/c intestines compared to C57BL/6 (Fig. 1A). The largest difference was observed for *aldh1a1*, which is known to be highly expressed by epithelial cells lining the gut [36, 37]. Remarkably, when expression of *aldh1a1* was analyzed along the gut axis, starting at the stomach, we observed the highest expression in the proximal SI, while expression decreased toward the distal part of the SIs (Fig. 1B). In all three parts of the SI, *aldh1a1* expression was significantly higher in BALB/c mice compared to C57BL/6 mice. These data are in line with earlier published results, showing differences of *aldh1a2* expression and ALDH activity in DCs along the gut axis, with a higher expression in the SIs compared to the colon [38–40]. Of note, while *aldh1a* mRNA was higher expressed in BALB/c mice compared to C57BL/6 mice, the serum retinol levels in both mouse strains were not significantly different (Fig. 1C).

Since retinoic acid receptor β (Rarβ) is a known direct target gene of RA-mediated signaling, *rarβ* mRNA expression levels can be viewed as an indicator of the RA-mediated signaling level that is taking place [41, 42]. Analysis of *rarβ* showed that mRNA levels were significantly increased in BALB/c SI and colon compared to C57BL/6, indicating a higher level of RA-mediated signaling within the intestines of BALB/c mice (Fig. 1D), while retinol serum levels were not different. Since a relation between GM-CSF-mediated signaling and *aldh1a* expression in DCs has been suggested in literature [43], we also checked whether *GM-CSF* levels were different within the SIs of the two mouse strains. No significant difference could be found (Fig. 1E). As a control, we analyzed *ifng* and *il4* mRNA levels, corrected for *cd3* mRNA expression, as markers for Th1- and Th2-skewing, respectively. As expected, *ifng* expression was significantly higher in C57BL/6 SIs, while the expression of *il4* mRNA was significantly higher in SI samples of BALB/c mice (Fig. 1F).
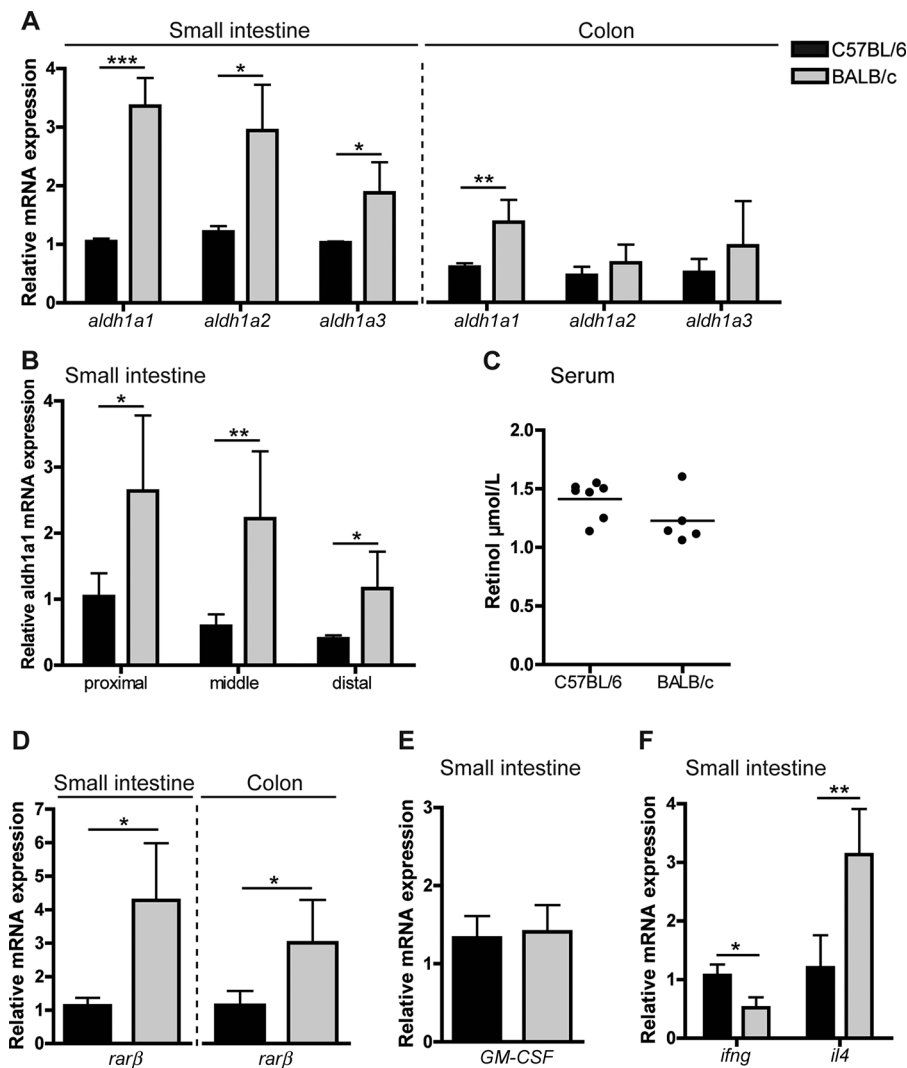
Figure 1. RALDH enzyme expression is increased in BALB/c small intestine (SI) and colon. Expression levels of *aldh1a1*, *aldh1a2*, *aldh1a3* mRNA, and (D) *rarβ* mRNA in SI and colon, respectively, were analyzed from C57BL/6 (black bars) and BALB/c (gray bars) by qPCR. (B) Expression of *aldh1a1* mRNA was analyzed in proximal, middle, and distal parts of the SI. (C) Retinol serum levels were determined in adult BALB/c and C57BL/6 mice and expressed in μmol/L. (E) Expression levels of *GM-CSF* mRNA were analyzed in SI. Expression of transcripts (A, B, D, E) was normalized to *ubiquitin C*. (F) Expression levels of *ifng* and *il4* mRNA were analyzed in whole SI samples from C57BL/6 and BALB/c by qPCR. Expression of transcripts was normalized to *cd3* mRNA levels. Relative mRNA expression levels in C57BL/6 SIs was set at 1.0 for all genes analyzed. (A–F) Data are shown as mean + SD of at least five mice per group and are representative of two independent experiments performed. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$; Student's *t*-test.

BALB/c MLN-DCs show higher (R)ALDH activity than C57BL/6 MLN-DCs

Studies have shown that CD103^{+} MLN-DCs represent a population of migratory DCs derived from the lamina propria, transporting orally derived antigen (Ag) from the intestine to the MLN and inducing gut-tropic T lymphocytes as well as Treg cells [44, 45]. It has been hypothesized that DCs acquire the mucosal phenotype within the intestinal environment, which is created by the epithelial cells. Indeed, we and others have shown that contact of BM-derived DCs with gut epithelial cells induced expression of RALDH enzymes and educated BM-derived DCs to induce gut-homing T cells in vitro [36, 46]. Furthermore, we have shown that RA directly induces RALDH enzyme activity in BM-DCs in vitro and that in the absence of dietary intake of vitamin A, RALDH activity is lacking in CD103^{+} DCs in MLNs [17]. We therefore tested whether the increased RA-mediated signaling observed in SIs of BALB/c mice could result in higher RALDH enzyme activity in CD103^{+} MLN-DCs when compared to C57BL/6 mice. RALDH activity was measured using the ALDEFLUOR assay, a flow cytom-

etry based assay with a fluorescent substrate that is specific for aldehydedehydrogenase enzymes. Flow cytometry plots of ALDE-FLUOR and CD103 showed that most of the ALDEFLUOR signal could be observed within the CD103^{+} DC population of MLNs in both BALB/c and C57BL/6 mice (Fig. 2A), confirming an earlier report [13]. In addition, analysis of CD103^{+} ALDEFLUOR expressing DCs revealed that BALB/c mice had significantly more CD103^{+} DCs with ALDH activity compared to C57BL/6 within the MLN (Fig. 2B). This activity was ALDH specific, since ALDEFLUOR expression was highly decreased in presence of ALDH inhibitor DEAB, while no difference between BALB/c and C57BL/6 could be observed under these conditions (Fig. 2B).

BALB/c mice display enhanced B-cell IgA class switching and IgA secretion into the intestinal lumen

Due to their highest *aldh1a2* expression levels, CD103^{+} MLN-DCs are now being recognized as the DC subset that is best equipped to induce RA-mediated signaling in both T and B cells,
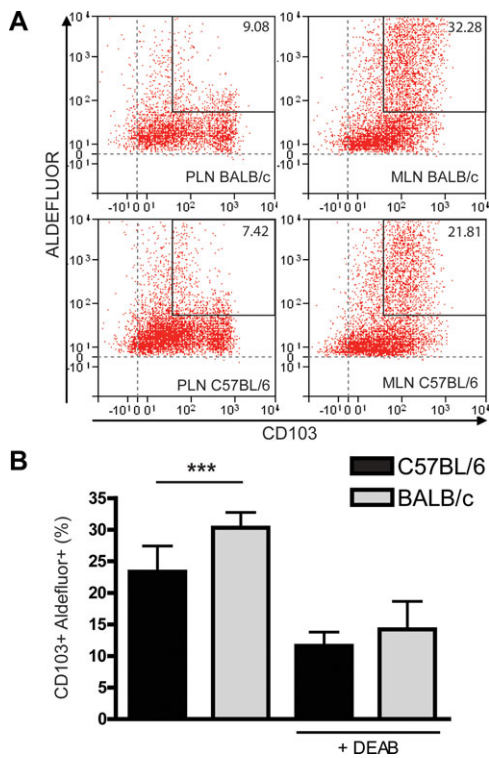
Figure 2. RALDH activity is increased in MLN-DCs from BALB/c mice. MHC-II$^+$CD11c^{high} DCs in peripheral LN (PLN) and MLN cell suspensions from C57BL/6 (black bars) and BALB/c (gray bars) mice were analyzed for CD103 expression and RALDH activity using the ALDE-FLUOR assay. (A) Representative FACS plots ($n = 10/2$ groups) are shown for ALDEFLUOR signal and CD103 expression by MHC-II$^+$CD11c$^+$ DCs in PLN and MLN. (B) Graph shows percentages ALDEFLUOR-positive CD103$^+$MHCII$^+$CD11c$^+$ MLN-DCs in absence or presence of RALDH inhibitor DEAB. Data are shown as mean + SD ($n = 10$ mice) and are pooled from two independent experiments. ***$p < 0.005$; Student's t-test.

Figure 3. BALB/c mice secrete higher levels of IgA into the small intestine lumen. Concentration of secretory IgA (μg/mL of PBS-dissolved feces) in feces isolated from (A) BALB/c mice (gray bars) and C57BL/6 mice (black bars), or (B) from C57BL/6 mice receiving either RA orally for 7 days or vehicle control, measured by ELISA. (C) FACS data representing percentage of IgA-positive B cells after 6 days of B-cell cultures without (gray bars) or with MLN DCs (black bars) from C57BL/6 or BALB/c mice. (D) Total production of IgA (in ng/mL) by B cells from coculture with MLN DCs (gray bars) and production as percentage of IgA-positive B cells as shown in Fig. 3C (black bars). (E) IgA production in cocultures of BALB/c B cells and DCs in the absence or presence of the Rar antagonists LE 135/540. Data are shown as mean + SD of at least six mice per group, and are representative of two independent experiments performed. *$p < 0.05$, ***$p < 0.005$; Student's t-test, except for (C) two-way ANOVA.

leading to FoxP3 T-cell differentiation, the induction of gut-homing molecules, and IgA class switching in B cells [13, 20, 47–49]. To address whether indeed BALB/c mice displayed higher RALDH activity in MLN-DCs when compared to C57BL/6, we tested whether the induction of IgA class switching in B cells and the production of secretory IgA into the intestinal lumen would differ between BALB/c and C57BL/6 mice.

First, we checked the production of secretory IgA in the feces of the SI of BALB/c and C57BL/6 mice, which was measured by ELISA. We observed that the concentration of IgA within the feces of BALB/c mice was remarkably higher compared to C57BL/6 mice (Fig. 3A). Moreover, when C57BL/6 mice were given extra RA, IgA concentrations were significantly increased in the feces of the SI (Fig. 3B). To test whether this was indeed a result of increased RA-mediated signaling, we analyzed the capacity of MLN-DCs from both strains to induce splenic B cells to become IgA-producing cells. Hereto, splenic B cells and MLN-DCs were brought into culture for 6 days. In the absence of MLN-DCs, no significant differences in percentages of IgA-positive B cells from C57BL/6 and BALB/c mice could be observed (Fig. 3C). Upon addition of MLN-DCs, a significant increase of IgA-positive B cells could be
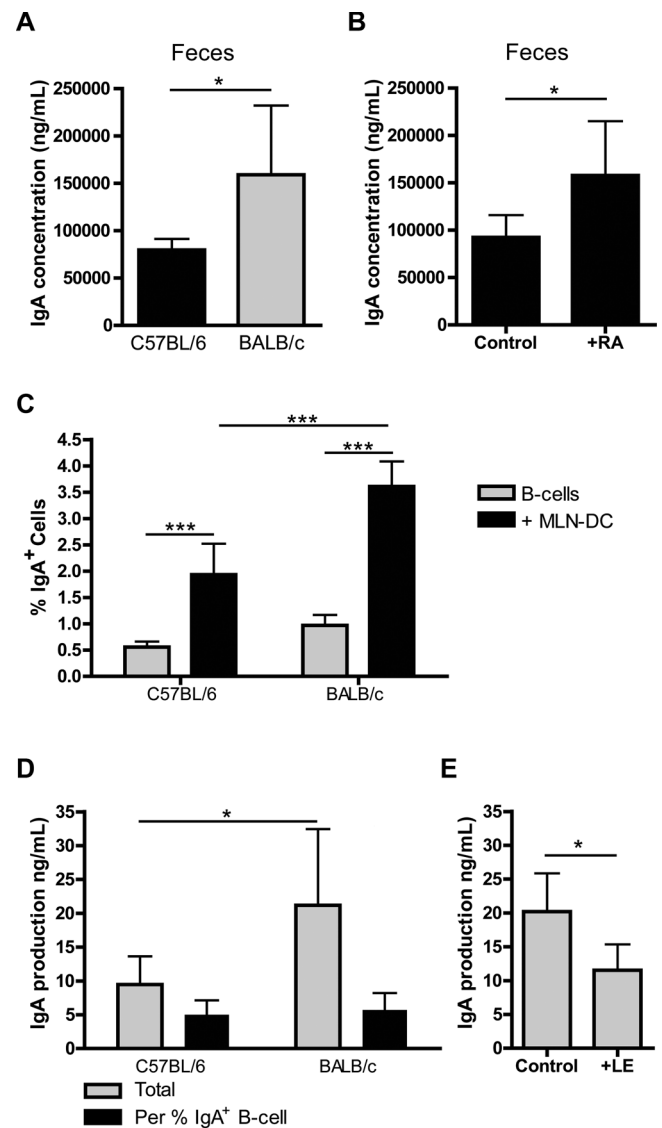
observed in both conditions. The increase of IgA-positive B cells was significant higher in BALB/c B cells compared to C57BL/6 B cells (Fig. 3C). In addition, higher levels of IgA protein were detected within the supernatant of BALB/c cocultures compared to C57BL/6 cocultures, further confirming the enhanced induction of IgA-producing B cells by BALB/c MLN-DCs (Fig. 3D). Upon

calculation of the produced IgA protein per IgA^{+} B cells, no differences in the production of IgA per IgA-expressing B cell could be observed in the two mouse strains (Fig. 3D). Furthermore, when we performed the same coculture with BALB/c cells in presence of the Rar antagonist LE 135/540, a decreased IgA production was observed (Fig. 3E). This demonstrates the necessity of RA secretion by DCs for the induction of IgA production by B cells, confirming data obtained in C57BL/6 mice [20].

Together our data showed that BALB/c MLN-DCs were better equipped, in an RA-dependent manner, to induce IgA class switching in B cells compared to C57BL/6 MLN-DCs, while the amount of IgA protein produced per B cell was similar.

Enhanced accumulation of lymphocytes in BALB/c intestines

As BALB/c MLN-DCs are better equipped to induce IgA class switching, we reasoned that other RA-dependent processes induced by MLN-DCs, such as the induction of gut-homing molecules, may occur differently in BALB/c mice when compared to C57BL/6 mice. However, it has been reported that the induction of gut-homing molecules depends on the Ag dose, while additionally OVA specific CD4^{+} transgenic T cells from BALB/c mice were shown to recognize overlapping as well as dissimilar OVA epitopes when compared to C57BL/6 mice [49, 50]. This precluded the use of these transgenic T cells to address whether T-cell activation by oral administration of OVA lead to differential induction of gut-homing molecules. We could however address whether more efficient homing of lymphocytes to the SI lamina propria had occurred in adult BALB/c versus C57BL/6 mice during normal homeostasis. For analysis of hematopoietic cells by FACS, CD45^{+} cells needed to

be positively selected in order to separate them from the mucus. This, however, excluded the possibility to obtain a measure of absolute numbers of hematopoietic cells within BALB/c versus C57BL/6 intestines, while it allowed analyses of B and T cells within the CD45^{+} populations. We found no differences in the percentages of B and T cells within the total CD45^{+} population from the SIs between C57BL/6 mice and BALB/c mice (Fig. 4A). To further analyze whether the absolute number of hematopoietic cells differed between both mouse strains, the expression levels of *cd45* (hematopoietic cells), *cd3* (marker for T cells), and *cd19* (marker for B cells) were analyzed in the intestines using real-time PCR. Data showed that the expression of *cd45* mRNA in BALB/c SI and colon was increased about twofold compared to C57BL/6 (Fig. 4B). In addition, mRNA levels for *cd3* and *cd19* were significantly higher in BALB/c SIs and colon when compared to C57BL/6 SIs (Fig. 4B). These data agree with an earlier report that administration of all trans-RA before immunization resulted in increased expression of both CCR9 and α4β7 in C57BL/6 mice [51]. Collectively, our results demonstrate that increased numbers of lymphocytes are present within the intestines of BALB/c mice compared to C57BL/6 mice.

BALB/c SIs contain more FoxP3^{+} T cells

In addition to the essential role of RA in the induction of gut-homing molecules on lymphocytes, RA has also been reported to be involved in the differentiation of FoxP3-expressing CD4^{+} Treg cells [14, 17, 18]. We therefore investigated the presence of CD4^{+} FoxP3^{+} T cells in SI and MLN of both C57BL/6 and BALB/c mice. FACS analysis of intracellular FoxP3 expression by CD4^{+} T cells showed that the percentage of FoxP3^{+} cells within the CD4^{+} population was not different in MLNs from BALB/c mice
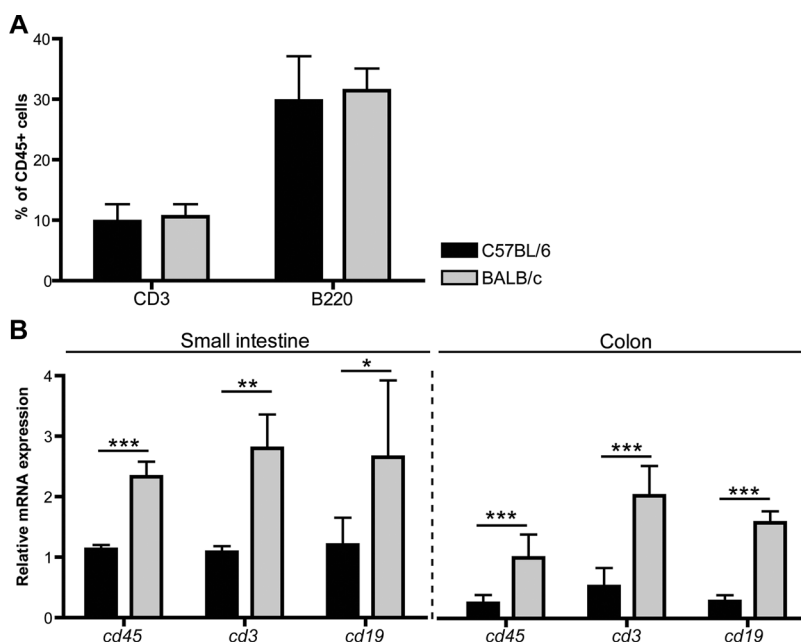

Figure 4. Enhanced accumulation of lymphocytes in intestinal lamina propria of BALB/c mice. FACS analysis of single-cell suspensions of total small intestines (SIs) from C57BL/6 and BALB/c mice that were stained for B220 and CD3. (A) Percentages of CD3^{+} T cells or B220^{+} B cells in total pool of CD45^{+} cells from SI of C57BL/6 (black bars) and BALB/c (gray bars) mice. (B) Expression of *cd45*, *cd3*, and *cd19* mRNA was analyzed in whole SI and colon, respectively, by qPCR. Expression of transcripts was normalized to *ubiquitin C*. Relative expression levels in C57BL/6 SIs were set at 1.0 for each gene analyzed. Data are shown as mean + SD of at least five mice per group and are representative of two independent experiments performed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$; Student's t-test.
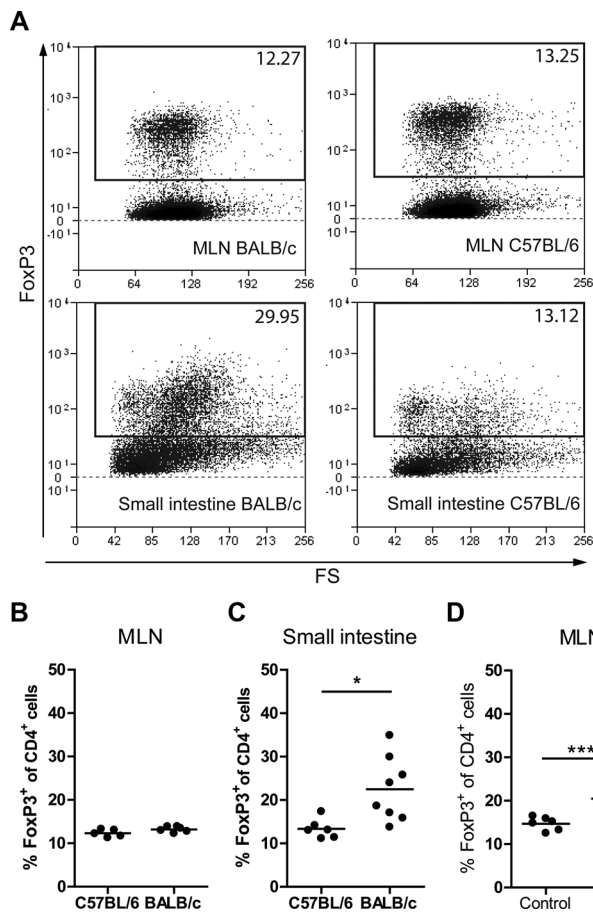
Figure 5. Higher percentage CD4+ T cells are FoxP3 Treg cells in BALB/c small intestines (SIs). Single-cell suspensions of MLNs and SIs from C57BL/6 and BALB/c mice were stained for CD45, CD4, and FoxP3 and analyzed by flow cytometry. (A) Representative FACS plots ($n = 6/2$groups) of FoxP3 from MLN (top) and SI (bottom) of BALB/c (left) or C57BL/6 (right) CD4+ T cells. Data represent percentage of FoxP3+ cells among CD4+ T cells of individual (B) MLN samples and (C) SI samples of minimum six mice per group from two independent experiments. (D) SI samples from C57BL/6 control and RA-treated mice. Data are shown as mean + SD of six mice per group from one experiment. $^*p < 0.05$, $^{***}p < 0.005$; Student's t-test.
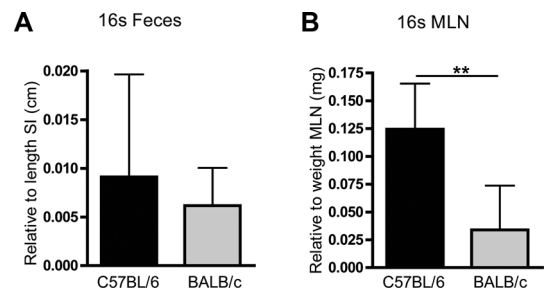

Figure 6. Higher bacterial translocation to MLN in C57BL/6 mice. (A, B) Bacterial 16s DNA measured by qPCR in feces and MLN of C57BL/6 (black bars) and BALB/c (gray bars) correlated to length of the small intestine and weight of the MLN, respectively. Data are shown as mean + SD of six mice per group from one experiment. $^{**}p < 0.01$; Student's t-test.

BALB/c mice have less bacterial 16S DNA within the gut draining MLN

Lymphocytes within the intestinal lamina propria are needed for proper homeostasis, while in particular secretion of IgA into the lumen of the intestines forms a barrier against commensal bacteria and invasive pathogens. Since C57BL/6 mice had lower levels of IgA in the feces when compared to BALB/c mice, we measured whether more bacteria had translocated across the epithelial barrier in C57BL/6 mice. No differences could be found in the levels of bacterial 16s DNA within the feces of both mouse strains, when correlated to the length of the SIs (Fig. 6A). However, higher levels of bacterial 16s DNA per milligram tissue could be detected in MLN derived from C57BL/6 mice compared to BALB/c MLNs (Fig. 6B). These results are indicative of a reduced protective barrier allowing higher levels of bacterial translocation to C57BL/6 MLN when compared to BALB/c mice.

Vitamin A decreases the severity of chronic colitis

To further address the physiological consequences of the differences in vitamin A metabolism observed in BALB/c versus C57BL/6 mice, we analyzed the role of vitamin A in a DSS-induced colitis model. As it has been reported for these mouse strains that they display comparable clinical symptoms when given different levels of DSS, we reproduced these models in our facility [30–35]. Indeed, only when BALB/c mice received 5% DSS, the severity of the disease was most comparable to C57BL/6 mice receiving 2% DSS when colon length, inflammatory score, and diarrhea score were measured (data not shown).

To investigate if the differences of RA-mediated processes have an effect on the severity of colitis, C57BL/6 mice were raised on a diet containing either normal amounts of vitamin A (vitamin A control diet) or on a diet lacking vitamin A (vitamin A deficient diet). When the animals were 10 weeks of age, colitis was induced by the administration of 2% DSS in the drinking water for 5 days. Vitamin A deficient mice suffered from a more severe colitis than mice on a vitamin A control diet as indicated by the weight loss. In fact, five of the seven animals included in the experiments had to be euthanized before the end of the experiment because of

compared to C57BL/6 mice (Fig. 5A and B). However, the percentage of FoxP3+ cells within the CD4+ population in SIs from BALB/c mice was significantly higher when compared to C57BL/6 mice (Fig. 5C). Moreover, upon oral administration of RA for 7 days, C57BL/6 mice exhibited an increased percentage of FoxP3-positive cells among CD4+ cells within the MLN (Fig. 5D). In addition, it was previously demonstrated that BALB/c mice, fed on a vitamin A low diet, had reduced percentages of FoxP3+ T cells in the SIs, which could be rescued upon i.p. administration of RA for 7 days [24]. Our data and the published data together reveal a critical role for RA to the percentages of FoxP3+ T cells found in the mucosal immune system. In conclusion, higher RALDH activity of CD103+ DCs in BALB/c mice correlated with higher percentages of FoxP3+ Treg cells in SIs.

A

C57BL/6 Vitamin A control diet

B

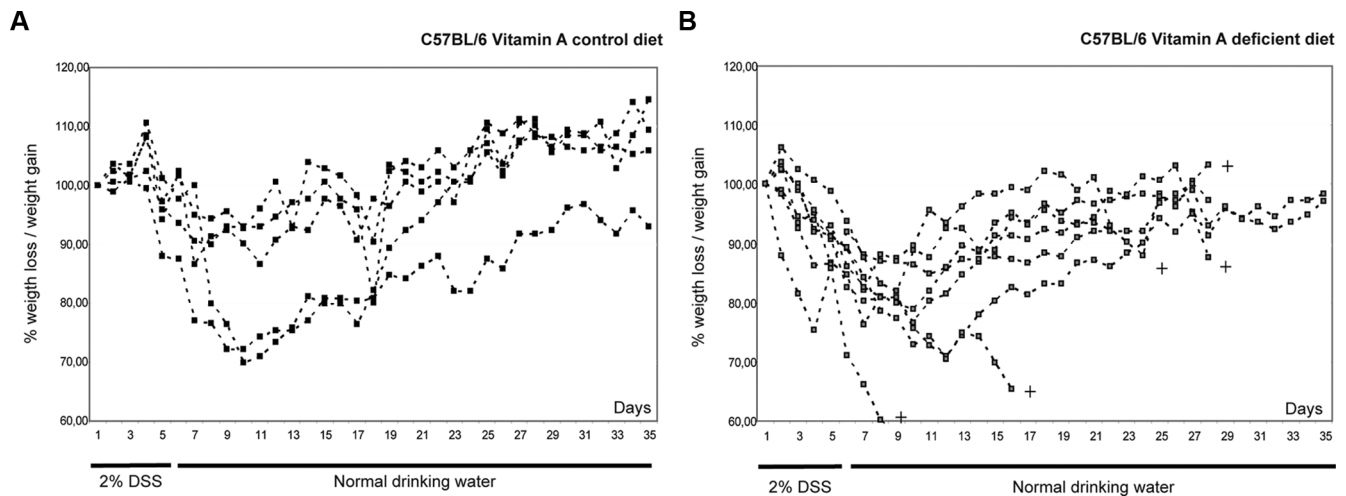C57BL/6 Vitamin A deficient diet

Figure 7. Vitamin A consumption has a beneficial effect in colitis. (A and B) Daily weight graphs of C57BL/6 mice on (A) a vitamin A control diet (VAC) and (B) a vitamin A deficient diet (VAD) given 2% DSS in drinking water for the first 5 days of the experiment followed by a 30-day period on normal drinking water. Per group seven animals were used.

severe disease. Only the remaining two mice returned to their starting weight at the end of the experiment, whereas the all vitamin A control mice recovered and returned to their starting weight (Fig. 7A and B). These data further support the notion that levels of vitamin A metabolism determine the extent to which the host is protected against damage within the intestines as seen during inflammatory bowel diseases.

Discussion

In this study, we demonstrated that BALB/c mice displayed an enhanced vitamin A metabolism, since *aldh1a* expression levels and RA-mediated signaling in SI and colon were higher when compared to C56BL/6, as well as RALDH activity in CD103$^+$ MLN-DCs, while serum retinol levels were comparable.

We observed increased mRNA levels for T and B cells in the intestines of these mice as well as a higher number of FoxP3$^+$ Treg cells, while comparable numbers of FoxP3$^+$ T cells were observed within the MLN. Increased numbers of B and T lymphocytes within the lamina propria could be a result of the enhanced capacity of BALB/c MLN-DCs to induce gut-homing molecules on activated lymphocytes. Also, the enhanced number of FoxP3$^+$ cells within BALB/c SIs could be the result of higher expression levels of gut-homing molecules on these cells but could also be caused by a more efficient differentiation due to higher RA levels or both. That indeed BALB/c MLN-DCs have the intrinsic capacity to more efficiently induce RA-mediated immune functions was shown by their ability to induce more IgA class switching on B cells when compared to C57BL/6 MLN-DCs. Consequently, increased production of secretory IgA was found within the lumen of the SI in BALB/c mice compared to C57BL/6 mice. Although we have tried to measure direct levels of RA in both LNs and lamina propria of the two different mouse strains with the use of tandem mass spectrometry [52], the levels were too low to obtain reliable data. However, it has already been shown that during embryogenesis, the levels of

RA-producing enzymes correlate with the levels of RA itself [53], indicating that the observed differences in RALDH expression and activity provide reliable information for RA levels.

Since RA-mediated signaling is crucial in shaping the mucosal immune system, it becomes important what the underlying cause of the observed differences between BALB/c and C57BL/6 is. We suggest that the difference in RALDH1 expression in intestinal epithelial cells contributes to the differences in RALDH2 activity in CD103$^+$ DCs [36, 39, 46]. Additionally, it has been published that GM-CSF is an important factor involved in imprinting of RALDH expression in mucosal DCs [43]. However, we observed no significant difference in *GM-CSF* mRNA levels in BALB/c and C57BL/6 SIs, suggesting that this does not contribute to the observed differences in RALDH expression levels in BALB/c versus C57BL/6 (Fig. 1E). As the difference in RALDH1 expression consequently affects the functioning of the mucosal immune system, it will be of interest to determine the underlying cause of the differential RALDH1 expression in these mouse strains. As serum retinol levels are not affecting the RALDH1 levels within the intestines [17], underlying genetic differences must contribute to the differential RALDH1 expression.

We propose that enhanced RA-mediated signaling seen in BALB/c mice when compared to C57BL/6 mice may lead to a more efficient mucosal immune system. Consequently, BALB/c may combat mucosal associated pathology better. We and others have shown that BALB/c mice are more resistant to developing colitis and require a higher dose of DSS compared to C57BL/6 mice to induce a comparable disease severity [30–35]. These differences have been attributed to a difference in Th1–Th2 balance in these mice, since BALB/c mice are known as prototypical Th2-type mice, while C57BL/6 mice show a more Th1-driven response. In addition, treatment with RA improved human and murine colitis by increasing the number of Treg cells [54, 55]. We propose here that variation in RA metabolism might determine disease susceptibility in these mice, since RA affects homing of lymphocytes to the intestines as well as

isotype switching toward IgA. Moreover, RA availability benefits epithelial integrity and increases the barrier function of the intestinal epithelial cells [56, 57]. In addition, RA was recently shown to affect the differentiation and cytokine production of intestinal innate lymphoid cells (ILCs) toward IL-22-producing group 3 ILCs, which protect mice against DSS-induced colitis [58, 59]. These data suggest that the differentiation of ILCs and their cytokine production within the lamina propria of BALB/c mice might be different when compared to C57BL/6 mice, as they have a higher vitamin A metabolism.

Confirming the effects of vitamin A and RA in our study, we showed that removal of vitamin A from the diet, thereby decreasing the RA levels, gave rise to a more severe form of colitis. This could lend an explanation as to why BALB/c mice are more resistant to DSS-induced colitis. Moreover, it has been shown by Bai et al. that BALB/c mice undergoing trinitrobenzene sulfonic acid induced colitis showed less severe colitis when they additionally received all-trans-RA. The data correlated with inhibition of Th1 cytokines and enhanced production of the Th2 cytokines IL-4 by lamina propia mononuclear cells [60]. Interestingly, in the context of the human form of disease, it has been shown that inflammatory bowel disease patients frequently have lower levels of serum vitamin A [61].

Although BALB/c mice are more resistant to developing DSS-induced colitis, these mice are more susceptible compared to C57BL/6 when combating infections that require a typical Th1-type response, such as *L. major* infection in the skin. Resistant C57BL/6 T lymphocytes produce IFN-γ that activates macrophages to produce NO and kill the parasite, while susceptible BALB/c T lymphocytes instead produce more IL-4 that suppresses macrophages [62, 63]. Therefore, BALB/c mice show enhanced mucosal immune system at the expense of the peripheral immune system.

Since enhanced RA-mediated signaling may lead to a decreased Th1–Th2 balance, increased induction of FoxP3^{+} Treg and epithelial barrier function, modulation of RA availability would be beneficial to increase the mucosal immune response and resistance to infections and immune diseases. Studies in vitamin A deficient patients showed indeed a compromised Th2-type immune response and a cytokine imbalance skewed toward Th1 [64].

In conclusion, our data suggest that the enhanced RA synthesis in BALB/c mice, when compared to C57BL/6 mice, results in the development of a more efficient mucosal immune system. Control of RALDH levels may therefore be an attractive way to direct the mucosal immune response.

Materials and methods

Mice

Specific pathogen free C57BL/6 mice and BALB/c mice aged 10–14 weeks were obtained from Charles River (Charles River, Maastricht, the Netherlands). Generation of vitamin A deficient or control animals was based on a method previously described by Iwata et al. [16]. Two different custom-made diets based on modified AIN-93M with the use of vitamin-free casein (MP Biomedicals, Solon, OH, USA) were used: vitamin A deficient diet containing no vitamin A and vitamin A control diet containing 2800 IU/kg vitamin A. Furthermore, C57BL/6 mice were fed with global 16% protein rodent diet (Harlan, Horst, the Netherlands), supplemented with 100 µg RA per gram dry food or vehicle control for 7 days (Sigma-Aldrich, Zwijndrecht, the Netherlands).

For the induction of DSS-induced colitis, we made use of a previously described single-dose DSS colitis model by Melgar et al. [31]. C57BL/6 mice were given 2% DSS for the first 5 days in their drinking water ad libitum, which was changed on a daily basis, after which normal drinking water was provided for the subsequent 30 days.

Mice were kept under standard animal housing conditions. Mice receiving defined vitamin A diet and mice in which colitis was induced were kept in individual ventilated cages. The Animal Experiments Committee of the VU Medical Center approved all of the experiments described in this study.

Preparation of SI cell and LN suspensions

SIs were dissected and opened longitudinally after removal of Peyer's patches. SIs were washed with HBSS without Ca^{2+} and Mg^{2+} containing 15 mM HEPES and 250 µg/mL gentamicin (all Invitrogen, Breda, the Netherlands) to remove fecal contents. Small intestinal segments were incubated twice with HBSS containing 5 mM EDTA (Sigma-Aldrich, Zwijndrecht, the Netherlands), 15 mM HEPES (Invitrogen), 10% FCS (HyClone Laboratories/Greiner Bio-One, Alphen aan den Rijn, the Netherlands), 1 µM DTT (Promega Benelux, Leiden, the Netherlands), and 14 mM 2-ME (Sigma-Aldrich) for 15 min at 37°C while constantly stirring to remove mucus. Pieces of SI were further cut with scissors and digested at 37°C for 20 min, using 150 µg/mL Liberase Blendzyme 2 (Roche, Penzberg, Germany) and 200 µg/mL DNAse I (Roche) in HBSS containing 15 mM HEPES and 10% FCS, while constantly stirring. Cell suspensions were subsequently immunomagnetically purified for CD45^{+} cells with PE-Cy7-labeled anti-CD45 (clone 30-F11; eBioscience/Immunosource, Halle-Zoersel, Belgium) and the EasySep PE positive selection kit (StemCell Technologies, Grenoble, France). Purified CD45^{+} lamina propria cells were used as cell suspensions for flow cytometry and analyzed with a Cyan ADP flow cytometer (Beckman Coulter, Mijdrecht, the Netherlands).

Single-cell suspensions were made by cutting LNs with scissors, followed by digestion at 37°C for 25 min while constantly stirring, using 0.5 mg/mL Blendzyme 2 (Roche) and 0.2 mg/mL DNAse I (Roche) in PBS.

Flow cytometry and ALDEFLUOR assay

RALDH activity in cells was measured using the ALDEFLUOR assay kit (StemCell Technologies), according to the manufacturer's

protocol. For flow cytometric analysis of ALDEFLUOR-reacted cells, cells were subsequently stained with anti-CD11c-PE (clone N418, eBioscience, Immunosource, Halle-Zoersel, Belgium), anti-CD103-biotin (clone M290, BD Bioscience, Breda, the Netherlands), anti-MHC-II-647 (clone M5/114), and with Sytox Blue (both Invitrogen) to discriminate between live versus dead cells. Secondary antibody used was Percp-conjugated (BD Bioscience) streptavidin. In addition, small intestinal and MLN cells were stained with anti-CD3ε-488 (clone 145-2C11), anti-FoxP3–647 (clone FJK-16s, both eBioscience), and B220 (clone CD45R). Antibody MHC-II was affinity-purified from hybridoma cell culture supernatants with protein G-Sepharose (Pharmacia, Uppsala, Sweden), stainings were performed with FoxP3 staining set (eBioscience). Cells were analyzed with a Cyan ADP flow cytometer (Beckman Coulter).

Coculture of splenic B cells with LN-DCs

B cells were positive selected from a single-cell spleen suspension using EasySep Mouse PE Positive Selection Kit (StemCell Technologies) with B220-PE (eBioscience). MLNs were collected from the same mice and DCs were isolated using CD11c beads with the autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. Cells were cultured in a 1:1 ratio for 6 days in IMDM medium, supplemented with 10% v/v heat-inactivated FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL) (all Invitrogen) together with 10 μg/mL goat F(ab')2 anti-mouse IgM (Southern Biotech, Birmingham, USA) for B-cell activation. A total of 10 μM of Rar antagonist LE 135 (Tocris Bioscience) and LE 540 (Wako Chemicals) was added to the culture. Supernatant was used for measurement of IgA production. For analysis of cell surface IgA expression, the cells were collected and analyzed by flow cytometry using biotin conjugated anti-IgA (Southern Biotech).

Enzyme-linked immunosorbent assay for secretory IgA

Feces from the SIs of mice were collected in cold PBS buffer. Debris was removed by cold centrifugation for 20 min at 2000 rpm in order to harvest the supernatant for analysis of secretory IgA. Supernatant of MLN DCs and B-cell cocultures were used directly for ELISA. Plates were coated with anti-mouse-IgA antibody to capture secretory IgA or mouse-IgA used as a standard (clone s107), followed by anti-mouse-IgA-biotin (all Southern Biotech) antibody, and subsequently streptavidin labeled with HRP. Samples were analyzed with a Fluostar Optima microplate reader (BMG Labtech, Isogen Lifescience, De Meern, the Netherlands).

RNA/DNA isolation and qPCR

SIs and colons were dissected from C57BL/6 and BALB/c mice, flushed with PBS, and homogenized in TRIZOL (Gibco, Invitrogen). RNA was isolated by precipitation with isopropanol. cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer's protocol. For genomic bacterial DNA isolation, MLNs and feces samples were collected and lysed with proteinase K (Qiagen, Germany). Bacterial DNA was isolated according to *Wilson's* protocol [65] and qPCR was performed. Specific primers for *cd45, cd3, cd19, csf2* (*GM-CSF*), *il4, ifng, rar*β, *aldh1a1, aldh1a2, aldh1a3, 16s,* and primers for housekeeping gene *ubiquitin C* (Isogen Life Science; Invitrogen) were designed across exon–intron boundaries using Primer Express software (PE Applied Biosystems, Foster City, CA) and Vector NTI software (Invitrogen), (see Table 1 for primer sequences). qPCR analysis was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Total volume of the reaction mixture was 10 μL, containing cDNA, 300 nM of each primer, and SYBR Green Mastermix (PE Applied Biosystems).

Table 1. Primer sequences used for real-time PCR

Gene	Forward primer	Reverse primer
ubq	AGCCCAGTGTTACCACCAAG	ACCCAAGAACAAGCACAAGG
cyclo	ACCCATCAAACCATTCCTTCTGTA	TGAGGAAAATATGGAACCCAAAGA
cd45	CCCCGGGATGAGACAGTTG	AAAGCCCGAGTGCCTTCCT
cd3	GTGGCTACTACGTCTGCTAC	TGGACTGTCGTCATCGGTATT
cd19	GTGCTCTCCCTTCCTACATC	CTGACCTTCTTCTTCCCCTC
csf2	AAACTCAAGGGCGCCTTGA	CTTGTGTTTCACAGTCCGTTTCC
il-4	GAGCTCGTCTGTAGGGCTT	GACTCATTCATGGTGCAGC
ifnγ	TACTACCTTCTTCAGCAACAGC	AATCAGCAGCGACTCCTTTTC
rarβ	GCCTGCAGAAGTGCTTTGAAGT	GCTCTCTGTGCATTCCTGCTTT
aldh1a1	CTCCTCTCACGGCTCTTCA	AATGTTTACCACGCCAGGAG
aldh1a2	TCATCAAAACCCTGAGGTATTATGC	GGGCTCGTGTCTTGTGAAAGTAA
aldh1a3	GTGTGCTTCACCAGGCATGA	CACAGGGCAGGAGCCAGTT
16s	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG

Statistics

Results are given as the mean $\pm$ SD. Statistical analyses were performed using the two-tailed Student's *t*-test with [*] $p < 0.05$, [**] $p < 0.01$, [***] $p < 0.005$, or when stated otherwise.

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