Transcription factor ISX mediates the cross talk between diet and immunity

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The intestinal epithelium is a major site for the conversion of dietary β-carotene to retinaldehyde by the enzyme BCO1. The majority of retinaldehyde is further metabolized to retinol (vitamin A), esterified and packaged into triacylglycerol-rich chylomicrons for bodily distribution. Some serve on-site for the synthesis of retinoic acid, a hormone-like compound, which exerts pleiotropic and dominant effects on gastrointestinal immunity. We report here that the intestine-specific homeobox protein ISX is critical to control the metabolic flow of β-carotene through this important branching point of vitamin A metabolism. This transcription factor represses *Bco1* gene expression in response to retinoic acid signaling. In ISX-deficient mice, uncontrolled *Bco1* gene expression led to increased retinoid production in the intestine. Systemically, this production resulted in highly elevated hepatic retinoid stores. In the intestine, it increased the expression of retinoic acid-inducible target genes such as *Aldh1a2*, *Dhrs3*, and *Ccr9*. The β-carotene–inducible disruption of retinoid homeostasis affected gut-homing and differentiation of lymphocytes and displayed morphologically in large lymphoid follicles along the intestine. Furthermore, it was associated with an infiltration of the pancreas by gut-derived lymphocytes that manifested as a pancreatic insulitis with β-islet cell destruction and systemic glucose intolerance. Thus, our study identifies an important molecular interlink between diet and immunity and indicates that vitamin A homeostasis must be tightly controlled by ISX to maintain immunity and tolerance at the intestinal barrier.

carotenoids | retinoids | lymphocytes | intestine | BCO1

Dietary lipids impact many aspects of mammalian biology (1). As a classic example, the vitamin A metabolite all-*trans*-retinoic acid (RA) is critical for organogenesis, cell differentiation, and immunity (2, 3). Because animals cannot synthesize this lipid from endogenous metabolites, precursor molecules must be acquired by the intestinal epithelium from the diet. These precursors exist in the diet either as preformed vitamin A, mainly retinyl esters (RE), or provitamin A carotenoids, mainly β-carotene (BC). Ultimately, all naturally occurring retinoids in the food chain are metabolically derived from carotenoids, and BC is a major source for these compounds in the human diet (4, 5).

The content of provitamin A in natural food sources is variable and is subject to seasonal fluctuations (4). Recently, we described an intrinsic regulatory mechanism, which helps mammals cope with this fluctuation. Central to this regulation is the RA-inducible transcription factor ISX that is expressed in epithelial cells of the intestine (6, 7). ISX suppresses gene expression of the scavenger receptor class B type 1 (*Scarb1*) and the β-carotene-15,15′-dioxygenase (*Bco1*), which encode proteins that respectively mediate the uptake of carotenoids and their conversion into retinoids (8–10). This negative feedback regulation controls the utilization of dietary BC for retinoid production in mice depending on demand and availability (9, 10).

Common single-nucleotide polymorphisms in *ISX*, *BCO1*, or *SCARB1* genes affect serum BC levels in humans, suggesting that the role of ISX in the control of intestinal BCO1 activity is well conserved (11). However, several studies indicate that the ISX protein may serve additional functions (6). For instance, this transcription factor has been implicated in cell proliferation and inflammatory responses (12, 13). Moreover, genetic polymorphisms in the *ISX* gene have been associated with inflammatory bowel disease in genome-wide association studies (14). Therefore, a common denominator for the transcription factor's putative dual role in vitamin A metabolism and immunity remains to be defined.

In the gastrointestinal tract, absorbed dietary BC is metabolized to RE for transport and bodily distribution, but also can be metabolized to RA, which via nuclear receptor binding can elicit a large spectrum of on-site immune responses (15). Previously, it has been shown that unbalanced supplies of dietary vitamin A can increase susceptibility to infectious diseases (16, 17), promote inflammation (18, 19), and can result in loss of tolerance against food antigens (20). Thus, we speculated that ISX lies at an important intersection of vitamin A metabolism and immunity and plays a critical role in the homeostatic control of these processes. To explore this putative role of the transcription factor, we examined ISX-deficient mice under various dietary conditions. We observed several indicators that retinoid metabolism and gastrointestinal immunity was compromised in these animals. The pathology was exacerbated with BC supplementation and associated with inflammatory processes.

Results

***Isx* Genotype Affects Intestinal Immune Homeostasis.** To define the role of ISX in gastrointestinal immunity, we analyzed the intestines of 7-mo-old $Isx^{-/-}$ and $Isx^{+/+}$ (wild-type) mice. We first measured mRNA expression levels of key enzymes of intestinal

Significance

The intestine is the major entrance site for nutrients, electrolytes, and water while constituting an effective barrier against toxins, antigens, and enteric flora. Hence, the intestinal mucosa harbors one of the largest populations of lymphocytes in the body. Nutrients such as vitamin A have been recognized to influence gut homing and differentiation of these cells. We identified the ISX protein as a critical molecular mediator of this cross talk between diet and immunity. This transcription factor regulates vitamin A production from dietary precursor molecules. Loss of this control in mice disrupts vitamin A homeostasis and impairs immunity. Reported genetic polymorphisms in the *Isx* gene have been associated with inflammatory disorders in humans, indicating that our findings in mice have clinical implications.

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vitamin A metabolism such as *Bco1* and the retinal dehydrogenase 2 (*Aldh1a2*). In $Isx^{-/-}$ mice, we observed a greater than 200-fold increase in mRNA levels of *Bco1* in the jejunum compared with controls (Fig. 1*A*). Expression levels of the RA-forming enzyme *Aldh1a2* were not significantly altered between genotypes (Fig. 1*B*). To directly assess biochemical consequences of ISX deficiency, we performed HPLC analyses for retinoid composition in lipid extracts from the jejunum. These analyses revealed that all-*trans*-retinol (ROL) and RE levels increased in ISX-deficient animals compared with controls (Fig. 1*C*). Alterations in retinoid metabolism of ISX-deficient animals also were mirrored in significantly higher levels of RE in hepatic stores of $Isx^{-/-}$ mice compared with wild-type mice (Fig. 1*D*).

To examine whether the *Isx* genotype affected immune cell differentiation, we determined the mRNA expression levels of the chemokine receptor *Ccr9* in a total RNA preparation of the jejunum. This chemokine receptor is expressed in gut-homing T and B cells in intestinal lymph follicles in response to RA signaling (21). We observed a significant increase in *Ccr9* mRNA levels in the jejunum in $Isx^{-/-}$ mice compared with $Isx^{+/+}$ mice (Fig. 1*E*). To discern if changes in *Ccr9* gene expression were accompanied by cellular alterations, we performed histology. ISX-deficient mice displayed an overall normal stratification of the intestinal cell layers (Fig. 1*F*). However, several regions in the duodenum and jejunum of these animals displayed large follicles. Microscopic inspection revealed that these structures were filled with mononuclear cells which morphologically resembled lymphocytes (Fig. 1*G*).

ISX Is Critical to Control Intestinal β-Carotene Absorption and Conversion to Retinoids. We next determined whether phenotype of ISX-deficient mice was associated with altered intestinal retinoid metabolism. Thus, we challenged $Isx^{-/-}$ and $Isx^{+/+}$ mice with BC supplementation. Upon a 6-wk dietary intervention, $Isx^{-/-}$ mice showed a 27-fold higher level of RE in the jejunum than $Isx^{+/+}$ mice (Fig. S1 *A* and *B*). Similarly, jejunal ROL levels were significantly increased in these mice (Fig. S1 *A*–*C*). The increased utilization of the provitamin for retinoid production was echoed in high levels of BC in the jejunal lipid extracts of $Isx^{-/-}$ mice (Fig. S2 *A* and *B*). Conversely, $Isx^{+/+}$ displayed a significantly higher amount of nonabsorbed BC in the feces compared with $Isx^{-/-}$ mice (Fig. S2*C*). Increased vitamin A production was also observed in highly elevated body stores of the vitamin in liver (Fig. S1*D*). LC-MS analysis of jejunal lipid extracts revealed that RA levels were fourfold higher in $Isx^{-/-}$ mice compared with $Isx^{+/+}$ mice (Figs. S1*E* and S3). Accordingly, expression levels of *Dhrs3* mRNA were increased in the jejunums of $Isx^{-/-}$ mice (Fig. S2*D*). This short-chain retinal dehydrogenase converts retinaldehyde to ROL, and its expression is induced by retinoid signaling (22). Moreover, we observed an increase of the expression of the RA-catabolizing enzyme *Cyp26a1* in liver of $Isx^{-/-}$ mice compared with $Isx^{+/+}$ mice (Fig. S2*E*). Thus, we concluded that ISX is required to maintain vitamin A homeostasis in response to dietary BC.

Dietary β-Carotene Affects Intestinal Immune Cell Differentiation During Adolescence. We next analyzed whether dietary BC can exacerbate the immune phenotype of $Isx^{-/-}$ mice. For this purpose, we randomized isogeneic $Isx^{-/-}$ littermates. One group received a diet supplemented with BC, while the other group received the same diet without BC supplement. After 10 wk of dietary intervention, supplemented mice had highly increased intestinal retinoid levels, whereas levels in nonsupplemented littermates were comparable to wild-type mice (Fig. 2*A* and Fig. S1). We also observed an increase of the expression levels of *Aldh1a2* (Fig. 2*B*). The expression of this gene is controlled by feed-forward regulation via RA in the intestinal mucosa (23). Histological analyses revealed that large lymphoid follicles were distributed along the entire small intestine in supplemented $Isx^{-/-}$ mice (Fig. 2 *C* and *D*), while in nonsupplemented $Isx^{-/-}$ mice, few large lymphoid follicles were detectable in the ileum (Fig. 2*C*). Immunofluorescence microscopy demonstrated the accumulation $CD45^{+}$ cells in the lymphocyte follicles of $Isx^{-/-}$ mice supplemented with BC (Fig. 2*E*). The stimulatory effects of BC supplementation on gastrointestinal immunity of ISX-deficient mice were further confirmed by a fourfold increase of *Ccr9* mRNA expression levels in jejunal RNA preparations (Fig. 2*F*). Because ISX has been associated with inflammation (12, 13), we also evaluated the expression levels of the tumor necrosis factor α (TNFα) gene which was fivefold higher in BC-supplemented $Isx^{-/-}$ mice compared with nonsupplemented littermates (Fig. 2*G*).

Maternal β-Carotene Supplementation Increases the Number and Size of Intestinal Lymphoid Follicles in ISX-Deficient Offspring. Dietary vitamin A is delivered via the maternal blood stream to the embryo (24) and affects lymphocyte differentiation in the developing gastrointestinal tract (25). Therefore, we wondered whether ISX might be a molecular linker between maternal vitamin A and immunity of the offspring. Thus, we subjected ISX-deficient dams to BC supplementation. As controls, we employed $Isx^{-/-}$ dams, which received regular mouse breeder chow rich in preformed

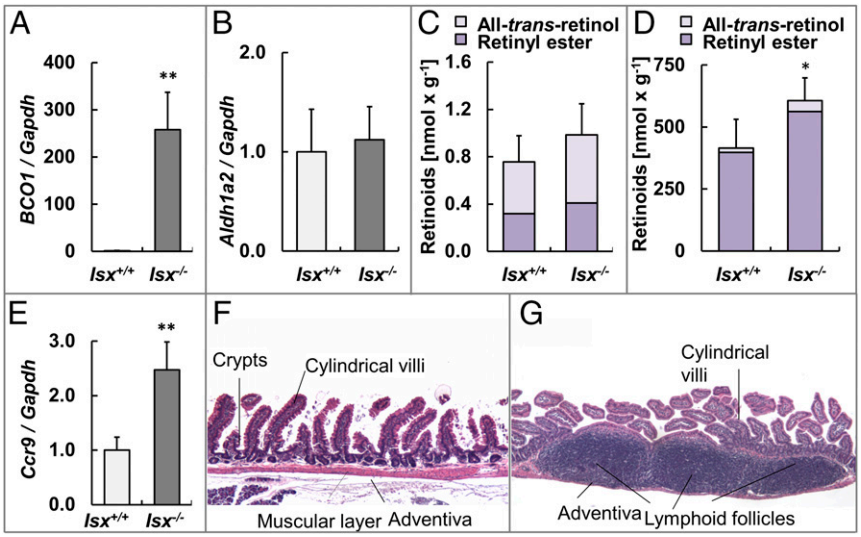

Fig. 1. ISX-deficient mice display enlarged lymphoid follicles in the small intestine. (*A* and *B*) Quantitative RT-PCR analysis of jejunal *Bco1* and *Aldh1a2* mRNA levels normalized to *Gapdh*. (*C* and *D*) Levels of nonpolar retinoids (all-*trans*-retinol and retinyl ester) in jejunal (*C*) and hepatic (*D*) lipid extracts. (*E*) Quantitative RT-PCR analysis of jejunal *Ccr9* mRNA levels normalized to *Gapdh*. (*F* and *G*) Representative H&E-stained cross sections through the small intestinal wall of a $Isx^{+/+}$ (*F*) and a $Isx^{-/-}$ mouse (*G*). Values indicate mean ± SD of results from at least five animals per genotype. Threshold of significance was set at $^{*}P < 0.05$ and $^{**}P < 0.01$. (Magnification: 20×.)

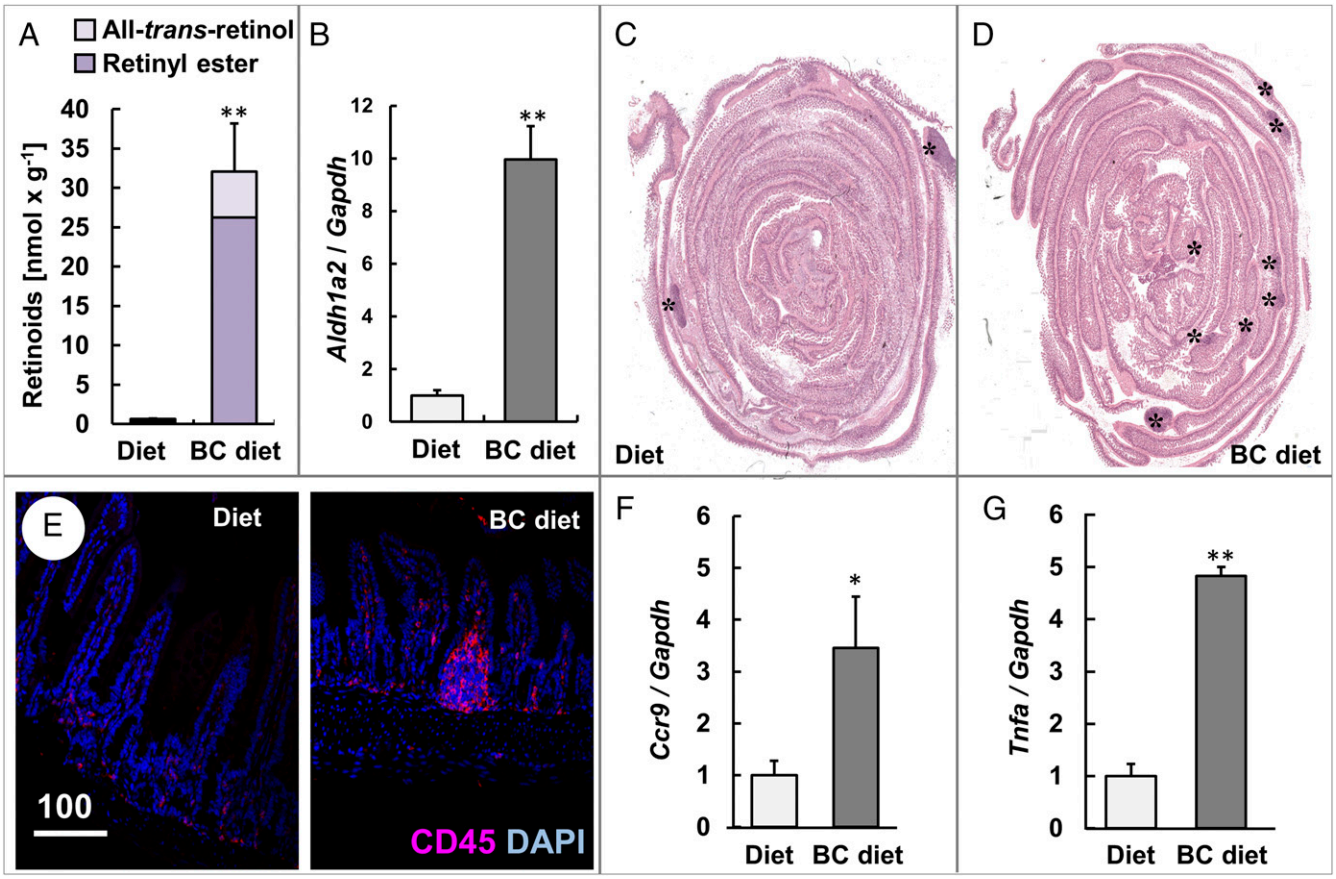

Fig. 2. β-Carotene supplementation increases the number of lymphocyte follicles, lymphocyte marker protein, and the proinflammatory cytokine. Four-week-old $Isx^{-/-}$ mice were fed with either diet or β-carotene (BC) supplemented diet. (*A*) Retinoid content of the intestines. (*B*) Quantitative RT-PCR analysis of jejunal *Aldh1a2* mRNA levels. (*C* and *D*) Representative H&E-stained cross section through the intestine. The asterisks (*) indicate the location of lymphoid follicles. (Magnification: 20×.) (*E*) Intestinal cross section immunostained for CD45 (red). Nuclei are stained with DAPI (blue). (Scale bar, 100 μm.) (*F* and *G*) Quantitative RT-PCR analysis of jejunal (*F*) *Ccr9* and (*G*) *Tnfa* mRNA levels. Values in *A*, *B*, *F*, and *G* indicate mean ± SD of results from five animals per supplementation group. Threshold of significance was set at $*P < 0.05$ or $**P < 0.01$.

retinoids. We obtained viable $Isx^{-/-}$ offspring both from BC-supplemented and nonsupplemented dams. At the age of weaning, we killed the offspring and analyzed their intestines. Large lymphoid follicles became detectable by macroscopic inspection in all parts of the intestine of offspring (Fig. 3*A*). Histological analyses confirmed the macroscopic observation and revealed an increase in the size and number of follicles in BC-supplemented offspring compared with nonsupplemented offspring (Fig. 3 *B*–*D*). We dissected the follicles from offspring of both supplementation groups and used flow cytometry to compare their lymphocyte composition. This analysis revealed significant overall increase of $CD45^+$ cells in BC-supplemented offspring (Fig. 3*E* and Fig. S4). Analyses of the T-cell ($CD3^+$) populations suggested alteration in the composition of $CD4^+$ and $CD8^+$ single-positive T cells and $CD4^-$ $CD8^-$ double-negative T cells in offspring of supplemented versus offspring of nonsupplemented dams (Fig. S4). To demonstrate that the immune phenotype was associated with altered vitamin A homeostasis, we performed HPLC studies for hepatic retinoids. Analyses of the two dietary groups revealed that liver stores of offspring of BC-supplemented dams displayed approximately double the amount of RE compared with offspring of nonsupplemented dams (Fig. 3*F*).

ISX-Deficient Mice Develop a Severe Insulitis. Recent work has indicated that diet is able to influence the immune system and thus can affect the development of inflammatory disease (26, 27). For instance, activated T helper cells expressing *Ccr9* have been detected in pancreatic islet lesions of the nonobese diabetic mouse model (28), and excess dietary vitamin A supplementation has been associated with loss of tolerance against common food antigens (20). We observed that immunity was altered in the intestines of ISX-deficient mice and that inflammatory markers were increased (Fig. 3). To analyze whether these mice developed any inflammation in the adjacent pancreas, we serially sectioned the organ. Pancreatic islets of wild-type mice displayed a circular shape with distinct borders to the surrounding cells as indicated by staining for insulin. In contrast, the islets of $Isx^{-/-}$ mice were irregularly shaped (Fig. 4*A*). To quantify the extent of the pancreatic pathology of ISX-deficient animals, we blindly scored the degree and occurrence of insulitis in serial sections of the pancreas. Two different cross sections of the whole pancreas (head to the tail) from 7-mo-old $Isx^{-/-}$ and $Isx^{+/+}$ mice ($n = 6$ each) were analyzed, and the degree of insulitis was scored according to the scale displayed in Fig. S5. From a total number of 282 islets that were examined in $Isx^{-/-}$ mice, 65% displayed the characteristic morphology of insulitis, with infiltrated immune cells (Fig. S5*B*). From these islets, 43% were identified as periinsulitis, 40% as mild insulitis, and 17% as severe insulitis. In contrast, only a few morphologically altered islets were detectable in pancreases of control mice. Out of 306 islets analyzed from $Isx^{+/+}$ mice, 23% displayed periinsulitis from which 84% were scored as an early stage of periinsulitis without altered eyelet morphology (Fig. 4*B*, third panel from the left). To further characterize the pancreas, we counted the total number of islets per pancreas. In this respect,

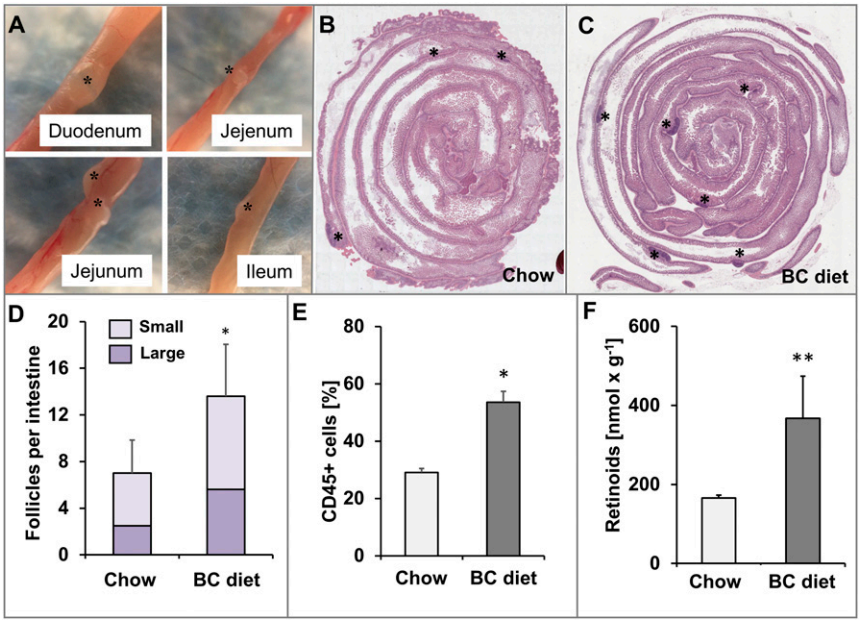

Fig. 3. β-Carotene supplementation of ISX-deficient dams increases the number and size of lymphoid follicles in the intestine of the offspring. Pregnant $Isx^{-/-}$ mice were fed with either chow or β-carotene (BC) supplemented diet, and 4 wk-old offspring were analyzed. (*A*) Macroscopic view of lymphoid follicles (asterisks) in different parts of the intestine of offspring. (Magnification: 12×.) (*B* and *C*) Representative H&E-stained cross sections through the entire intestine of offspring (duodenum is in the center). Lymphoid follicles are marked by asterisks. (Magnification: 20×.) (*D*) Quantification of the number of small and large lymphoid follicles in the intestine of offspring. (*E*) The percentage of $CD45^+$ cells in dissected intestinal lymphoid follicles of offspring. (*F*) Retinoid content of the liver of offspring. Values in *D* and *F* indicate mean ± SD of results from five animals, and values in *E* of four lymphoid follicles from two animals per supplementation group. Threshold of significance was set at $*P < 0.05$ or $**P < 0.01$.

$Isx^{-/-}$ mice had a significantly reduced average number of islets per pancreas compared with control mice (Fig. S5*C*).

Staining for CD45 confirmed that pancreatic islets of ISX-deficient animals were infiltrated with lymphocytes (Fig. 4*B*, arrows). Infiltration of the pancreas by gut-derived lymphocytes was further indicated by an increase of *Ccr9* mRNA expression in the total RNA preparations of the pancreas (Fig. 4*C*). Analysis for *Tnfa* mRNA expression showed that this maker of inflammation was 18-fold elevated in the pancreas of $Isx^{-/-}$ mice compared with controls (Fig. 4*D*). Since retinoids play a significant role in pancreas development and maintenance (29), we also determined their levels in the pancreas. We observed a slight increase in ROL and RE level in the pancreas of $Isx^{-/-}$ mice (Fig. 4*E*).

ISX-Deficient Mice Display Impaired Glucose Metabolism. To examine whether endocrine function of the pancreas was impaired in $Isx^{-/-}$ mice, we subjected animals to oral glucose tolerance tests (GTT). These tests revealed that $Isx^{-/-}$ mice displayed a reduced clearance rate of blood glucose after a bolus dose of glucose compared with sex- and age-matched control mice (Fig. S6*A*).

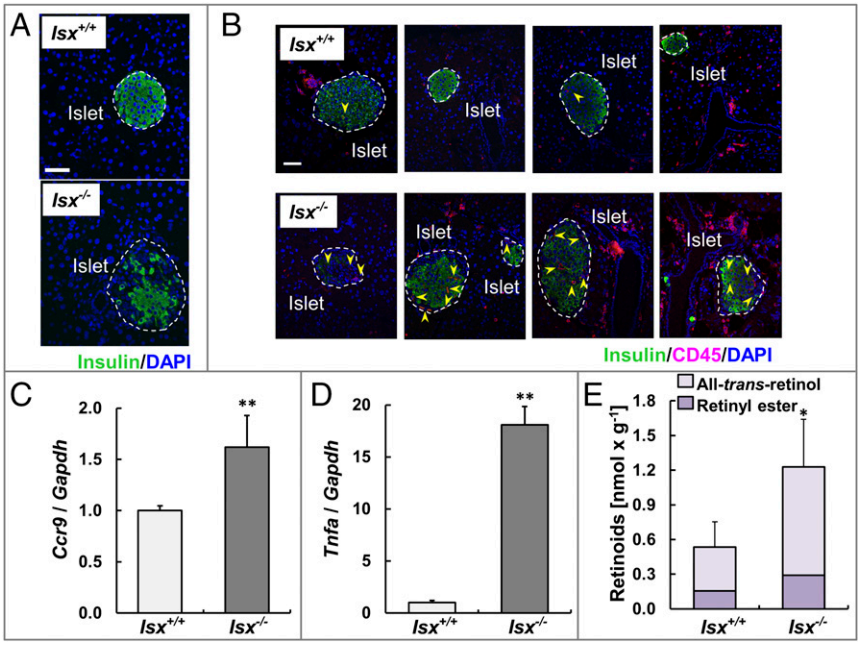

Fig. 4. ISX-deficient mice develop a pancreatic insulitis. (*A*) Immunostaining for insulin (green) of β-islets of 7-mo-old $Isx^{+/+}$ (*Top*) and $Isx^{-/-}$ (*Bottom*) mice maintained on standard diet. DAPI staining of nuclei is shown in blue. (Scale bar, 50 μm.) (*B*) Immunostaining of insulin (green) and CD45 (red) of representative pancreatic sections of these mice. DAPI staining of nuclei is shown in blue. The yellow arrows indicate CD45+ cells. (Scale bar, 50 μm.) (*C* and *D*) Quantitative RT-PCR analysis of *Ccr9* (*C*) and *Tnfa* (*D*) mRNA levels of these mice. (*E*) Retinoid content of the pancreas of these mice. Values in *C*–*E* indicate mean ± SD of results from five animals per genotype. Threshold of significance was set at $*P < 0.05$ or $**P < 0.01$.

Because vitamin A metabolism has been associated with insulin resistance of peripheral tissues (30), we also performed insulin tolerance tests. However, *Isx*$^{-/-}$ mice showed no difference in the level of blood glucose at each time point compared with control mice (Fig. S6*B*). This observation suggested that the *Isx*$^{-/-}$ genotype was not causing resistance to insulin in the periphery. To directly demonstrate that the endocrine pancreas function was impaired in ISX deficiency, we determined serum insulin after glucose administration. In this test, *Isx*$^{-/-}$ mice secreted lower amounts of insulin in the serum in response to glucose injection compared with respective *Isx*$^{+/+}$ control animals (Fig. S6*C*).

Dietary β-Carotene Induces Insulin Resistance in ISX-Deficient Mice. We showed that ISX-deficient mice developed a destructive pancreatic insulitis. If this pathology relates to intestinal vitamin A metabolism, one would expect that BC supplementation would expedite its occurrence. Thus, we performed GTTs with *Isx*$^{-/-}$ littermates raised on a diet supplemented with BC or the same diet supplemented with preformed retinoids as major sources of vitamin A. This analysis revealed that *Isx*$^{-/-}$ mice fed with BC for 10 wk had a reduced glucose clearance compared with siblings maintained on the retinoid-supplemented diet (Fig. 5*A*). Consistently, pancreatic sections of BC-supplemented mice displayed morphological alterations of β-islets in immunohistochemistry for insulin (Fig. 5*B*). Moreover, *Ccr9* mRNA expression levels were increased in BC-supplemented *Isx*$^{-/-}$ mice compared with littermates maintained on a retinoid-supplemented diet (Fig. 5*C*). Biochemical analyses of pancreas lipid extracts also unfolded increased retinoid levels in BC-supplemented animals compared with retinoid-supplemented controls (Fig. 5*D*).

Discussion

In the intestine, absorbed BC has a dual metabolic fate as a precursor for either RE or RA synthesis. We here provide evidence that a coordinated flux of dietary BC into these pathways evidently depends on regulation by the transcription factor ISX and is essential to maintain immunity and tolerance at this important barrier. Loss of ISX in mice affected retinoid metabolism in the intestine and also systemically. In the intestine, retinoids, including its transcriptionally active form RA, were significantly increased upon BC supplementation of ISX-deficient mice. This accumulation was largely prevented by ISX in the wild-type mice, which showed lower absorption and conversion rates of the provitamin. The enhanced absorption and conversion of dietary BC induced the expression of RA-inducible target genes such as *Aldh1a2*, *Dhrs3*, *Cyp26a1*, and the chemokine receptor *Ccr9*. This biochemical phenotype was associated with an increase in the number of intestinal lymphocytes, which accumulated in large lymphoid follicles. This phenotype was observed in aged ISX-deficient mice raised on diets with trace amounts of BC and was exacerbated by BC supplementation of the animals. The consequences of the ISX deficiency for immunity are consistent with previous studies which show that RA acts as an autacoid, a short-ranging hormonal signal (31), which can induce the expression of gut-homing receptors on T and B cells, promotes conversion of Foxp3^{+} regulatory T cells, and provokes T-cell–independent IgA switches in naïve B cells (21, 32, 33).

Our data also indicate that ISX plays a more general role for systemic retinoid homeostasis as related to immunity. In this context, it is important to note that RE from chylomicrons, the major transport mode of dietary vitamin A, can be transferred from the maternal circulation to the embryo (24). Additionally, existing evidence indicates that the maternal vitamin A status can imprint the size of secondary lymphoid organs and the efficiency of immune responses in the offspring (25). In keeping with a deterministic role of maternal vitamin A for the developing immune system, BC-supplemented ISX-deficient dams gave birth to pups with elevated hepatic retinoid stores and an increased number and size of secondary lymphoid organs along the intestine. These lymphoid follicles contained a larger number of CD45^{+} lymphocytes and displayed alterations in T-cell composition compared with offspring of dams which received RE instead of BC supplementation. Thus, our data indicate that ISX is an important molecular link between the diet and gastrointestinal immunity throughout the mouse life cycle.

An important question is why the utilization of dietary BC for retinoid production is restricted by ISX in mammals. At first glance, this issue is surprising because vitamin A is a limited nutrient, and its deficiency is associated with disease and mortality.

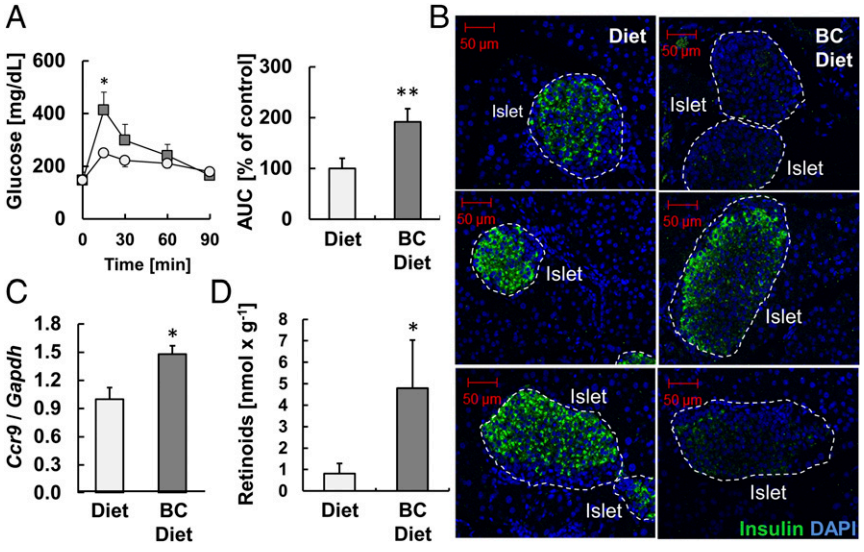

Fig. 5. β-Carotene supplementation impairs glucose metabolism in ISX-deficient mice. (*A*) Blood glucose levels from glucose tolerance tests of non-supplemented (circles) and β-carotene (BC) supplemented (squares) *Isx*$^{-/-}$ mice. (*Right*) The area under the curve (AUC) as percentage of control group. (*B*) Representative β-islets immunostained for insulin (green). DAPI staining of nuclei is shown in blue. (Scale bar, 50 μm.) (*C*) Quantitative RT-PCR analysis of *Ccr9* mRNA levels. (*D*) Retinoid (retinol and retinyl esters) levels of the pancreas of these mice. Values indicate mean ± SD of results from five animals per supplementation group. Threshold of significance was set at $*P < 0.05$ or $**P < 0.01$.

Accordingly, supplementation with preformed retinoids has been proven to be beneficial in malnourished populations (34). However, there is also evidence that excessive amounts of dietary vitamin A can trigger inflammatory responses (31, 35). Our studies in ISX-deficient mice indicate that dietary vitamin A has a narrow window of benefit for immunity. Thus, the utilization of BC, which exists in quite significant amounts in many fruits and vegetables (50–100 mg/kg), must be controlled to prevent the development of inflammatory diseases. As we report here, ISX-deficient mice developed a pancreatic insulitis that was associated with an infiltration of the pancreas by gut-derived lymphocytes. Though we did not clarify all molecular details, our data suggested that the loss of the ISX-dependent control of provitamin A metabolism triggered this pathology. This conclusion conforms to results from other studies, which demonstrate that excessive amounts of performed retinoids can promote inflammation and can trigger loss of tolerance at the intestinal barrier (20, 36). Such failure to establish tolerance against commensal flora and food antigens has been implicated in the autoimmunity that underlies type 1 diabetes (26). In keeping with this proposal, an invasion of activated T helper cells expressing *Ccr9* has been observed in pancreatic islet lesions of the nonobese diabetic mouse model (28). Similarly, we observed a pancreatic infiltration of $CD45^+$ cells and an elevation of *Ccr9* mRNA levels in total pancreatic RNA preparations of ISX-deficient mice. This phenotype was accompanied by highly elevated expression levels of *Tnfa* mRNA encoding an inflammatory marker of lymphocytes.

In conclusion, our study provided evidence that ISX plays an important role in maintenance of immunity and tolerance in response to diet-derived signals at the intestinal barrier. Our findings indicate that retinoid production from dietary BC must be tightly controlled to avoid disturbances in lymphoid immunity, which can trigger inflammatory responses in the gastrointestinal tract. In future studies, the ISX-deficient mouse will be a versatile model to study the molecular details of this intriguing interplay between diet and gastrointestinal immunity. A better understanding of the molecular factors which control mucosal immunity and tolerance will aid in the development of nutritional intervention strategies to improve health in neonatal and adult life.

Materials and Methods

Female $Isx^{-/-}$ and $Isx^{+/+}$ mice were generated as described (6, 9). All mice were on a C57/BL6;129Sv mixed genetic background. Mouse colonies were routinely maintained on breeder chow (29,000 IU vitamin A/kg diet). In supplementation experiments, mice were fed a diet based on AIN93G formulation (with 4,000 IU vitamin A/kg) without and with BC supplementation (50 mg/kg). HPLC analysis for BC and nonpolar retinoids was performed as previously described (10, 37). All-*trans*-retinoic acid was extracted, and LC-MS analysis was performed by a protocol adapted from Kane et al. (38). For qRT-PCR analysis an ABI Step-One Plus RT-qPCR instrument (Applied BioSystems) was used as previously described (10). For immunohistochemistry, protocols were applied as previously described (9). Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Scheffé tests using software origin 9 (OriginLab Corporation), with threshold of significance set as indicated in the figure legends. A detailed description of the materials and methods is provided in *SI Materials and Methods*.

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