Impaired Mucosal Immune Responses in Interleukin 4-targeted Mice

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Summary

Interleukin 4-targeted (IL-4-/-) mice are defective in T helper (Th2) cytokine production as determined after nematode infection. As Th2 cells appear to be selectively induced by oral immunization we investigated the ability of IL-4-/- mice to respond to perorally administered antigen. We found that IL-4-/- mice failed to respond to soluble protein antigens given perorally together with cholera toxin (CT) as a mucosal adjuvant. In contrast to wild-type mice no or poor anti-keyhole limpet hemocyanin (KLH) or anti-ovalbumin (OVA) B cell responses were observed in gut lamina propria, spleen, or serum of IL-4-/- mice after oral immunization. In addition, mucosal immunization failed to stimulate antigen-specific T cell responses in these mice. The lack of responsiveness was specific for mucosal administration of antigen and was not seen after intravenous injections with antigen and CT-adjuvant. The systemic adjuvant effect of CT was not impaired in IL-4-/- mice as evidenced by the strong enhancement of anti-KLH responses after intravenous immunization with KLH plus CT as opposed to KLH alone. However, CT as an immunogen, in contrast to KLH or OVA, stimulated significant mucosal and systemic immune responses in IL-4-/- mice after oral immunization. Both serum and intestinal IgA anti-CT antibodies were demonstrable in IL-4-/- mice as well as in wild-type mice. Total IgA levels in gut lavage and in serum of immunized IL-4-/- mice were of similar magnitude as in wild-type mice, suggesting that the ability of naïve B cells to undergo isotype switch-differentiation from IgM to IgA in IL-4-/- mice did not appear to be impaired. Immunohistochemical analysis of Peyer’s patches demonstrated a complete inability to form germinal centers in IL-4-/- mice in contrast to wild-type mice. Our data suggest that IL-4-/- mice are unable to respond to oral/mucosal immunization due to a failure to stimulate antigen-specific cells required to induce germinal center reactions in the Peyer’s patches. Our findings demonstrate that IL-4 and probably functional Th2 cells are required for induction of gut mucosal antibody responses.

It is widely believed that stimulation of gut mucosal immunity, and perhaps immunity at other mucosal surfaces, is best achieved by oral administration of antigen (1-2). However, in the healthy individual most soluble protein antigens given perorally are poorly immunogenic. More often proteins introduced in this manner, e.g., as food antigens, induce oral tolerance rather than immunity (3). Therefore, to improve the immunogenicity of mucosal vaccines, current research has focused on the regulatory mechanisms involved in gut mucosal immune responses and developing mucosal adjuvant systems (2, 4). Of the many interleukins associated with regulatory functions in the immune system, IL-4 has been demonstrated to play a central role (5). In particular, IL-4 may be of particular interest for the control of mucosal immune responses (8, 9). Moreover, IL-4 has been associated with T cell tolerance (10). In orally tolerized mice, increased levels of IL-4 mRNA in lymph nodes were reported suggesting that IL-4 also has a regulatory function on induction of tolerance following oral exposure to antigen (11). Finally, a role of IL-4 in IgA B cell differentiation has been indicated in recent studies (12-14).

Cholera toxin (CT), the enterotoxin of Vibrio cholerae, is perhaps the most potent mucosal immunogen yet described (2, 4, 15). It also acts as a strong mucosal adjuvant greatly

Abbreviations used in this paper: CMF, Ca²⁺- and Mg²⁺-free; CT, cholera toxin; HRP, horseradish peroxidase; LPL, lamina propria lymphocytes; MLN, mesentric lymph node; PNA, peanut hemagglutinin; PP, Peyer’s patches; SFC, spot-forming cells.
enhancing mucosal IgA and systemic IgG responses as well as preventing oral tolerization to unrelated soluble antigens administered together with CT perorally (16-19). Understanding the mechanisms of CT’s adjuvant and tolerance-breaking effects will be instrumental for the construction of future mucosal vaccines (1, 2, 4). Many immunomodulating and enhancing effects of CT on antigen presentation, B cell differentiation, and T cell functions have been reported, but it is still unclear which mechanisms are involved in the adjuvant function in vivo (2, 4, 20).

We recently reported that T cells of helminth-infected IL-4-/- mice exhibited an impaired Th2 response as demonstrated by reduced IL-5, IL-9, and IL-10 production (21). To address the role of Th2 cells in mucosal immune responses IL-4-/- mice were immunized with the highly immunogenic combination of KLH or OVA plus CT-adjuvant (16). We found that IL-4-deficient mice were greatly impaired in their ability to respond to either KLH or OVA with no or poor antigen-specific B or T cell responses detectable in the gut lamina propria, spleen, or serum. The poor mucosal antibody responses correlated with a failure to develop germinal centers in Peyer’s patches (PP) after oral immunizations in the IL-4-/- mice. Despite the poor antigen-specific mucosal responses, IL-4-/- mice appeared not to be impaired in the mechanism needed for IgA isotype switch as levels of total IgA and IgA containing cells in the small intestine were comparable to wild-type mice.

Materials and Methods

Animals. IL-4 gene-targeted mice (IL-4-/-) and control wild-type mice (IL-4+/+) were generated as described (21). The mice were maintained and bred at the Department of Medical Microbiology and Immunology (Göteborg, Sweden) in animal facilities under pathogen-free conditions using microisolator cages and sterile workbenches. All experiments were carried out with 8-12-wk-old mice of the same sex.

Immunizations. Oral immunizations with KLH (Sigma Chemical Co., St. Louis, MO) 2.5 mg/dose, or chicken egg albumin (OVA, grade V, Sigma Chemical Co.), 200 µg and 15 mg per dose, were given intragastrically alone or together with 10 µg per dose of cholera toxin (List Biological Laboratories, Campbell, CA), as described (19). Oral immunizations were given in 0.5 ml PBS containing 3% bicarbonate. The mice were immunized four times at 10-d intervals, and were killed 6 d after the final dose. Six mice were included in each group and samples were analyzed in pairs. Animals were immunized intravenously in the lateral tail vein with two doses, 10 d apart, containing KLH at 100 µg/dose in the presence or absence of CT at 1 µg/dose. Mice were killed 6 d after the final dose for further analysis as described above.

Preparation of Lymphoid Cells. Spleen and mesenteric lymph node (MLN) lymphoid cells were prepared by teasing the tissues through a nylon screen (16). Spleen red blood cells were subject to lysis by osmotic shock (16). Single cell suspensions were prepared and washed three times in HBSS (GIBCO, Paisley, Scotland) and diluted in Iscove’s medium (GIBCO) containing 10% FCS (GIBCO).

Intestinal lamina propria lymphocytes (LPL) were prepared as described (16). Briefly, after thorough washing in Ca²⁺- and Mg²⁺-free (CMF)-HBSS (GIBCO), the tissue pieces were incubated in CMF-HBSS containing 5 mM EDTA (Merck, Darmstadt, Germany) to remove epithelial cells and intraepithelial lymphocytes. The intestinal pieces were then incubated in three consecutive rounds of 60 min each with RPMI 1640 (GIBCO) containing 100 U/ml of collagene type C-2139 (Sigma Chemical Co.) to extract the LPL by enzymatic digestion. Finally, lamina propria cells recovered as single cell suspensions were washed twice in CMF-HBSS and resuspended in Iscove’s medium containing 10% FCS and adjusted to the appropriate cell density.

ELISPOT Assay. The lamina propria or splenic cells at 10⁶ cells/ml were analyzed for specific antibody production at the single cell level using the ELISPOT assay. Anti-KLH, anti-OVA, or anti-CT spot (antibody producing)-forming cells (SFC) activity/1⁶ lymphocytes were determined as previously described (16, 19). Briefly, small petri dishes (Nunc; Roskilde, Denmark) were coated at 4°C overnight with 100 µg/ml KLH, 200 µg/ml OVA, or 2 nmol/ml ganglioside GM1 followed by 3 µg/ml of CT. Single cell suspensions of lymphocytes at 10⁶ cells/ml in Iscove’s medium containing 10% FCS were added in 400 µl/petri dish and duplicate plates. Before SFC analysis, the petri dishes were blocked with 0.1% BSA in PBS at 37°C for 30 min. Cells were allowed to incubate at 37°C for 3.5 h. SFCs were visualized in a two-step process consisting of horseradish peroxidase (HRP)-labeled rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) at 1/200 dilution reacting at 4°C overnight followed by HRP-conjugated swine anti-rabbit Ig antibodies (DAKO) at 1/200 dilution for 2 h at room temperature, as an enhancing step. Spleen cells from individual mice or LPLs from two mice were pooled and analyzed in duplicates. The SFCs were developed by adding paraphenylenediamine (PPD), 0.5 mg/ml and 0.01% H₂O₂ in 1% agar in PBS as a thin film of substrate by pouring the prewarmed (46°C) solution into the dishes and then immediately discarding it. Antigen-specific antibody production in the lamina propria and spleen was expressed as total Ig SFC ± SD of each group.

Serum and Gut Lavage ELISA. The mice were bled before being killed. Gut lavage was obtained as described below. Immune sera and gut lavage were prepared and stored at -20°C until assayed by ELISA (16, 19). Briefly, polystyrene microtitre plates (Nunc) were coated with KLH (100 µg/ml) or OVA (200 µg/ml), or GM1 ganglioside (0.5 nmol/ml) (Sigma Chemical Co.) followed by CT (0.5 µg/ml) as described (16, 19). Sera or lavage at 1/50 or 1/10 dilution, respectively, were added and serial threefold dilutions were performed. The plates were incubated overnight at 4°C. Total Ig antigen-specific antibodies were demonstrated with HRP-conjugated rabbit anti-mouse Ig antibodies (DAKO) at 1/200 dilution and visualized using O-phenylenediamine (OPD) substrate (1 mg/ml)/0.04% H₂O₂ in citrate buffer (pH 4.5). The reaction was read in a Titertek Multiscan spectrophotometer (Flow Laboratories, Irvine, Scotland) at 492 nm. The anti-KLH, anti-OVA, and anti-CT titers were defined as the interpolated OD reading giving rise to an absorbance 0.4 above background. For analysis of antigen-specific antibodies of various isotypes after the addition of the sera and incubation overnight at 4°C, alkaline phosphatase-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE (Southern Biotechnology Associates, Birmingham, AL) were added at 1/500 dilution and incubated for 2 h at room temperature. Bound antibodies were visualized using phosphatase substrate tablets (NPP-tablets; Sigma Chemical Co.) at 1 mg/ml in ethanolamine buffer (pH 9.8). The reaction was read at 405 nm. The mice were analyzed individually and each serum sample was assayed in duplicates. ELISA values are given as serum or gut lavage log₁₀ titers expressed as means ± SD of each group. Determinations of total IgA were performed with 5 µg/ml of goat anti-mouse IgA coating
antibodies (Southern Biotechnology Associates) in PBS followed by incubations of samples and an IgA standard preparation (Phar-Mingen, San Diego, CA) at 400 ng/ml in serial twofold dilutions. As developing antibody we used the alkaline phosphatase conjugated goat anti-mouse IgA (Southern Biotechnology Associates) at 1/500 dilution.

Assessment of T Cell Priming. Spleen and MLN lymphocytes were extracted from IL-4−/− or IL-4+/+ mice orally primed with KLH or OVA alone or together with CT as described above. The lymphocytes were cultured at 10^6 cells/ml in Iscove's medium containing 10% FCS, gentamycin (50 μg/ml), and additional nutrients, in a volume of 1 ml/well in 24-well flat-bottomed plates (Nunc) at 37°C and 10% CO2 for 6 d. KLH or OVA antigen (Sigma Chemical Co.) were added to the wells at 100 and 1,000 μg/ml, respectively. For control purposes cells were cultured in medium alone without the addition of KLH or OVA-antigen. Supernatants were harvested on day 6 and frozen at −70°C until used. Supernatants were monitored for production of IFN-γ by ELISA, as described (19). The concentration of IFN-γ in each sample was calculated from the standard curve generated by rIFN-γ (Pan Data, Rockville, MD) and expressed in U/ml ± SD. The limit for detection of IFN-γ was 1–3 U/ml in this assay.

Immunohistochemistry. Frozen sections (5 μm) from unimmunized or from OVA and KLH plus CT-adjuvant primed IL-4−/− or wild-type mice were prepared on microslides using a cryostat (model 1720; Leitz, Wetzlar, Germany) and frozen at −70°C. The slides were fixed in 50% acetone for 30 s followed by 100% acetone for 5 min at 4°C and 5 min at room temperature. After washings in PBS, the slides were treated with 0.3% H2O2 for 5 min. For detection of IgA containing cells in gut mucosa, the slides were further treated with normal horse serum (1/20) in 0.1% BSA/PBS for 15 min in a humid chamber. Thereafter, FITC-conjugated goat anti-mouse IgA (Southern Biotechnology Associates) was added at 1/100 whereupon the slides were mounted on Aquatex (Merck) and photographed. For detection and analysis of germinal center formation in PP and MLN, fixed sections were double-labeled for the presence of IgM positive B cells vs. germinal center B cells using the following reagents: goat anti-mouse IgM (Southern Biotechnology Associates) and biotinylated peanut (Arachis hypogaea) hemagglutinin (PNA; Sigma Chemical Co.), followed by streptavidin-conjugated to FITC (Southern Biotechnology Associates). The sections were evaluated and photographed using an Axioskop microscope (Zeiss, Cambridge, UK).

Table 1. Impaired Local and Systemic Antibody Responses to Oral Immunizations in IL-4−/− Mice

<table>
<thead>
<tr>
<th>Table 1. Impaired Local and Systemic Antibody Responses to Oral Immunizations in IL-4−/− Mice</th>
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<tbody>
<tr>
<td><strong>Anti-KLH SFC/10^7 cells</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>Experiment</strong></td>
</tr>
<tr>
<td>I</td>
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<tr>
<td>II</td>
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<tr>
<td>III</td>
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<tr>
<td>IV</td>
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Mice were given four oral doses with KLH (2.5 mg/dose) with or without CT adjuvant (10 μg/dose) and killed 6 d after the final immunization. Spleen cells and gut lamina propria lymphocytes were prepared in single cell suspensions and analyzed for anti-KLH specific antibody-producing cells (SFC) with the ELISPOT assay. Values are given as means ± SD of three pairs of mice/group.
Table 2. Unaltered Splenic SFC Responses and Strong Adjuvant Effects of CT after Parenteral Immunizations in IL-4−/− mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Adjuvant</th>
<th>Anti-KLH spleen SFC/10⁷ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>No</td>
<td>IL-4−/− 728 ± 150</td>
</tr>
<tr>
<td>II</td>
<td>CT</td>
<td>IL-4−/− 2,658 ± 730</td>
</tr>
<tr>
<td>III</td>
<td>CT</td>
<td>IL-4−/− 2,375 ± 550</td>
</tr>
</tbody>
</table>

Mice were given two intravenous immunizations with KLH (100 µg) with or without CT adjuvant (1 µg) and killed 6 d after the final immunization. Single anti-KLH-producing cells (SFC) were detected by the ELISPOT assay. Values are given as means ± SD of three paired mice/group. Background activity in unimmunized mice is <100 SFC/10⁷ cells.

Table 3. Intact Anti-CT Responses to Oral Immunization with CT in IL-4−/− Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>anti-CT SFC/10⁷ cells</th>
<th>Serum total Ig anti-CT</th>
<th>Gut lavage IgA anti-CT</th>
<th>Percent antitoxic protection loop test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
<td>Lamina propria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−/−</td>
<td>350 ± 44</td>
<td>3,812 ± 578</td>
<td>3.1 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>+/+</td>
<td>743 ± 144</td>
<td>7,597 ± 947</td>
<td>3.7 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

Mice were given four oral doses with KLH (2.5 mg) plus CT (10 µg/dose) and killed 6 d after the final immunization. Serum (total Ig) and gut lavage (IgA) anti-CT antibody titers were analyzed by ELISA and given as mean log₁₀ titers of triplicates ± SD. Specific anti-CT-producing cells (SFC) were assayed by the ELISPOT method. Antitoxic resistance was determined by the intestinal loop protection assay using 2.5 µg/loop of CT for the challenge dose (16). Toxin-provoked intestinal loop test measuring fluid accumulation was expressed as weight/cm of small intestine and the protection afforded by prior oral immunization with CT was calculated and is given as percent protection relative to unimmunized mice. One representative experiment of three.
Table 4. Normal IgA Production in IL-4–/– Mice

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Gut lavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>–/–</td>
<td>200 ± 19</td>
<td>68 ± 24</td>
</tr>
<tr>
<td>+/+</td>
<td>218 ± 33</td>
<td>67 ± 18</td>
</tr>
</tbody>
</table>

Unimmunized IL-4–/– or +/+ mice were killed and the total IgA concentrations in serum and gut lavage were compared by ELISA. Five animals were included in each group and the values are expressed in μg/ml of IgA and represent the mean ± SD. The total IgA was calculated from a standard curve generated with purified mouse IgA from a hybridoma (PharMingen, San Diego, CA). This is one representative experiment out of three.

T cells from IL-4–/– mice after oral immunization (Fig. 1). In contrast, T cells from immunized wild-type mice gave significantly elevated IFN-γ responses to recall antigen (Fig. 1). Thus, our data demonstrate impaired ability to respond with specific B and T cells after oral immunizations in IL-4– targeted mice.

No Impairment of Parenteral Immune Responses and Intact Systemic Adjuvant Function of CT in IL-4–/– Mice. Since KLH administered perorally alone is unable to stimulate an immune response even in normal wild-type mice, the use of CT-adjuvant in these experiments was necessary (Table 1). Therefore, a poor mucosal response to oral immunization in IL-4–/– mice could be explained on the basis of lack of adjuvant effect of CT in IL-4–deficient mice. To rule out this possibility mice were immunized parenterally with KLH in the absence or presence of CT-adjuvant and the systemic anti-KLH responses were monitored. We found a strong enhancing effect of CT adjuvant on parenteral SFC responses to KLH in IL-4–/– as well as IL-4+/+ mice (Table 2). Moreover, in both groups serum anti-KLH IgG-responses were increased by 10-fold as a consequence of CT-adjuvant activity (not shown). In addition, systemic responses to KLH or KLH plus CT in IL-4–/– were of similar magnitude as in wild-type mice, suggesting that the defect to respond in IL-4–/– mice was restricted to mucosal immunization. The isotype distribution of anti-KLH antibodies after systemic immunization in IL-4–/– mice; i.e., no IgE, strongly decreased IgG1, unaltered IgM, and IgA and increased levels of IgG2a, IgG2b, and IgG3 (not shown), agrees well with earlier observations after conventional immunizations (21, 24). The result also argues against a direct effect of CT adjuvant on the isotype distribution in the KLH-specific response. Thus, the lack of mucosal immune responses to orally presented antigens in

determined by ELISA. (A) The data represent the mean log10 isotype-titers of three experiments ± SD. Three mice were analyzed individually/group. Values are given for serum anti-CT responses in IL-4–/– mice (open symbols) and IL-4+/+ (solid symbols). (B) Analysis of individual samples. Each symbol represents the titer of pooled serum from two mice. Anti-CT IgM and IgA mean log10 titers for IL-4–/– (circles) and IL-4+/+ (triangles) are given.

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IL-4−/− mice is likely to reflect a strong local requirement of IL-4 or Th2 cells for induction of mucosal immunity rather than a lack of adjuvant activity of CT in IL-4−/− mice.

Of note, no lamina propria anti-KLH SFC responses or anti-KLH gut lavage IgA responses were found in either IL-4−/− or wild-type mice after intravenous immunizations, indicating that systemic immunizations and specific serum IgA antibodies do not influence the intestinal IgA immune response (not shown).

In Contrast to Conventional Antigens CT Is Highly Immunogenic after Oral Immunization even in IL-4−/− Mice. It has been claimed that CTs adjuvant function is strictly associated with its own immunogenic properties (2, 4). In the following experiments we compared the response to KLH with that to CT itself after oral immunization with KLH plus CT. Interestingly, while KLH failed to stimulate significant immune responses, CT elicited both systemic and mucosal anti-CT SFC responses in IL-4−/− mice (Table 3). Moreover, the strong local anti-CT IgA response in the gut mucosa was associated with functional immune protection in that equally good resistance to challenge with CT of intestinal loops was observed in both IL-4−/− and wild-type mice (Table 3). However, the anti-CT antibody responses in IL-4−/− mice were between two- and threefold lower than in wild-type animals (Table 3).

The serum anti-CT isotype pattern of orally immunized IL-4−/− mice resembled the pattern found after parental intraperitoneal or subcutaneous immunizations in wild-type mice in that the dominance of IgG1 was shifted towards a dominance of IgG2a, IgG2b, and IgG3 without detectable IgE antibodies (Fig. 2, references 21, 22). Interestingly, we found a complete lack of serum anti-CT IgM responses in IL-4−/− mice whereas specific IgA antibody-levels were comparable with those detected in wild-type mice (Fig. 2). It is clear from these experiments that CT, probably because of its potent immunomodulating properties, can induce significant immune responses in mice that are otherwise impaired in their mucosal immune response.

For comparison we also analyzed anti-KLH responses in gut lavage in IL-4−/− and IL-4+/+ mice. In contrast to CT, we found that anti-KLH IgA responses after oral immunizations were undetectable in IL-4−/− mice, whereas wild-type mice had anti-KLH IgA titers comparable to the anti-CT IgA titers (Table 3), i.e., a log₁₀ titer of 1.6 ± 0.2 (not shown).

IgA B Cell Differentiation Appears Unaffected in IL-4−/− Mice. As illustrated in Table 3, IL-4−/− mice showed good IgA anti-CT responses in gut lavage and in serum after oral immunization. This finding suggested that despite the impaired ability to respond to conventional antigens given orally the IL-4−/− deficient state may not be associated with a failure to support B cell differentiation from IgM to IgA. Total IgA levels detected in gut lavage and in serum were found to be comparable in nonimmune IL-4−/− and +/+ mice (Table 4). Also, we investigated by immunohistochemical staining sections of small intestine for evidence of local IgA formation in the gut mucosa. We observed unaltered numbers of IgA containing plasma cells in the lamina propria of IL-4−/− mice as compared with wild-type mice (Fig. 3), suggesting that the absence of IL-4 (and probably Th2 cells) does not impair the ability to promote IgA differentiation.

The Impaired Mucosal Immune Response in IL-4−/− Mice Is Independent of Antigen Molecular Size or Dose. In a previous study we found that CT greatly increased gut permeability for luminal antigens (25). Since KLH is a relatively large molecule (300 kD) and CT is considerably smaller (86 kD), a possible explanation for the difference in mucosal immunogenicity in IL-4−/− mice might be ascribed to the difference in size. In the following experiment, using the same peroral immunization protocol as before, we replaced KLH with the much smaller antigen OVA, a 43-kD protein. Irrespective of OVA's smaller size, no immune response was detected in IL-4−/− mice after oral immunization, even with a relatively high dose of OVA (15 mg) plus CT (Table 5). In contrast, the wild-type mice responded with both mucosal as well as systemic anti-OVA antibody formation and showed evidence for good T cell priming, i.e., IFN-γ production by OVA-specific cells (Table 5). Furthermore, IL-4−/− mice exhibited impaired responsiveness to oral immunization with OVA irrespective of whether a high (15 mg) or a low (200 μg) dose was used together with the CT adjuvant (not shown). In wild-type mice the high dose of OVA gave a significant response whereas the low dose also did not stimulate a detectable anti-OVA response.

IL-4−/− Mice Fail to Develop Germinal Centers in PP. A possible explanation for an impaired intestinal immune response after oral immunization is the lack of PP, which are considered to be the inductive sites for gut mucosal immune responses (1). Gross inspection of intestines from IL-4−/− mice revealed a normal number and appearance of PP. Therefore, we hypothesized that the impaired mucosal immune response in IL-4−/− targeted mice might be secondary to an inability to stimulate specific T and B cells in the Peyer's patches. 10 d after a single oral priming dose of OVA admixed with CT adjuvant animals were killed and frozen sections were prepared from the small intestines. Immunohistochemical analysis for germinal center formation was then performed. As illustrated in Fig. 4, no germinal center formation was observed in the IL-4−/− mice, whereas IL-4+/+ mice exhibited prominent germinal center formation. B cells brightly labeling for both surface IgM and PNA were found exclusively in the wild-type mice, indicating the presence of germinal centers, while this staining pattern was absent in IL-4−/− mice.

Figure 3. Distribution of IgA containing cells in the lamina propria of unimmunized IL-4−/− mice (A) and IL-4+/+ mice (B). Sections of gut small intestine were incubated with anti-mouse IgA FITC-labeled antibodies (Southern Biotechnology Associates) and photographed at 40× original magnification using a Zeiss microscope.
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Recall antigen, OVA, for 4 d. IFN-γ values were determined by ELISA and are expressed in U/ml ± SD of triplicate cultures. Values are representative of at least three identical experiments.

Table 5. Impaired Local and Systemic B and T Cell OVA-specific Responses after Oral Immunization in IL-4−/− Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Spleen SFC/10^6 cells</th>
<th>Lamina propria OVA-T cell priming anti-OVA serum titers</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>−/−</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>0</td>
</tr>
<tr>
<td>+/+</td>
<td>925 ± 350</td>
<td>675 ± 450</td>
<td>3.3 ± 0.5 36.4 ± 3.0</td>
</tr>
</tbody>
</table>

Mice were given three oral immunizations with OVA (15 mg/dose) plus CT adjuvant (10 μg/dose) and killed 6 d after the final immunization. Serum anti-OVA antibody titers and anti-OVA antibody producing cells (SFC) were analyzed in IL-4−/− and wild-type (+/+ ) mice. Serum titers against OVA were determined as described in Materials and Methods. T cell priming was evaluated in spleen lymphocytes after culture with recall antigen, OVA, for 4 d. IFN-γ values were determined by ELISA and are expressed in U/ml ± SD of triplicate cultures. Values are representative of at least three identical experiments.

In contrast, germinal center formations were demonstrable in MLN in both wild-type and IL-4−/− mice. As illustrated in Fig. 5, cells brightly labeling with PNA (green) were detected in both mouse strains, suggesting that IL-4−/− mice are defective at the gut immune response PP inductive site while more systemic lymphoid tissues as MLN appear normal.

Discussion

This is the first study to demonstrate that IL-4-targeted mice are impaired in their ability to respond to orally presented antigens. The mechanism for the deficiency appears to be an inability to stimulate specific T and B cells in the PP, which are considered primary organs for induction of intestinal immune responses (1). We found that after oral priming with either KLH (not shown) or OVA in the presence of CT adjuvant, no germinal center formation was observed in PP of IL-4−/− mice, whereas these microenvironments were prominent in wild-type mice. We currently have no explanation for this deficiency. However, it is possible that IL-4 or additional factors produced by the Th2 type of CD4+ cells influence either the mechanism responsible for antigen uptake into the patch, or the events necessary for antigen triggering and activation of specific T and B cells in the PP. Recently, we reported that mice with a targeted disruption of the IL-6 gene (IL-6−/−) exhibited impaired mucosal immune responses (26). However, in contrast to the IL-4−/− mice, these mice also appeared to be severely impaired in B cell IgA differentiation (26). Although IL-6 is produced by Th2 cells, many other cells including macrophages, epithelial cells, and B cells are also capable of producing this cytokine (1). Moreover, previous studies have indicated that IL-6 affects IgA differentiation on already isotype committed B cells and therefore the impaired mucosal immune responses after local immunization in IL-4 and IL-6-deficient mice are most likely explained by different mechanisms. Preliminary experiments using oral immunizations with the KLH and CT adjuvant system in IL-6−/− mice have given results that would support such a notion.

Systemic immune responses in IL-4−/− mice were found to be of similar magnitude but to differ in isotype distribution as compared to those observed in wild-type mice. This finding agrees with previous reports showing no IgE and low IgG1 production and argues that in IL-4−/− mice B cells are provided with a different T helper activity as compared with wild-type mice (21, 24). The impaired mucosal response in IL-4−/− mice to oral immunization reflects the missing T helper activity uniquely required for induction of intestinal immune responses and perhaps mucosal responses in general. It has been suggested that mucosal immune responses are differently regulated as compared with systemic responses, although reports are conflicting (1). While Xu-Amano et al. (8, 9) have demonstrated the predominance of the Th2 cells in the gut immune system after oral immunization with SRBC or tetanus toxin, significant Th1 activity was most often also found by these investigators. We and others have previously found both Th1 and Th2 lymphokine production in mucosal tissues after oral immunization with strong production of both IL-4 and IFN-γ in lamina propria T cells (23, 27). It is not clear which of these two cytokine systems critically controls induction of mucosal immunity. The present study provides direct proof that IL-4 and possibly Th2 functions in general are required for the generation of mucosal antibody responses and T helper activities in the intestinal immune system. Whether IL-4 also is involved in the development of oral tolerance is currently not known. However, recent studies have indicated that IL-4 might play a role in tolerance and augmented IL-4 mRNA activity has been detected in orally tolerized animals (10, 11). We are currently investigating the relationship between active IgA immunity and oral tolerance in the IL-4−/− mice. Information provided by these studies might have important implications for future strategies in oral vaccine development.

It is well recognized that mucosal immune responses to orally delivered soluble protein antigens require an active ad-
juvant system (1, 2, 4). This is also illustrated in our study where we found no response to KLH alone after oral immunization (Table 1). Our results were obtained with CT as the adjuvant system and it might be argued that soluble protein antigens delivered together with a different adjuvant, independent of CT, might have stimulated a mucosal immune response in IL-4-/- mice. However, in preliminary experiments we observed that IL-4-/- mice orally immunized with OVA entrapped in microparticles also failed to respond whereas wild-type mice showed significant mucosal responses (our unpublished result). This result suggests that the impaired mucosal responsiveness in IL-4-/- mice extends also to particulate antigens and was not solely an inability to respond to the adjuvant effect of CT.

Most previous studies of the effects of CT have indicated strong inhibition of T cell functions (2, 4, 28, 29). Yet, CT is one of the most potent enhancers of immune responses known today (2, 4). For example, we have found that T cell priming in the presence of CT is greatly augmented and 20-40 fold increased frequencies of antigen-specific T cells were achieved as compared with immunizations with antigen alone (23). As recent in vitro studies of CT and other cAMP-increasing substances suggested that Th1 cells might be more susceptible to cAMP-mediated inhibition as compared with Th2 cells, a plausible explanation to CT's adjuvant effect might be that CT facilitates Th2 functions (30). We would then expect a lack of adjuvant function of CT in IL-4-/- mice that were shown to be defective for Th2 responses (21). To the contrary, we found that CT in systemic immune responses was both highly immunogenic and functioned as a strong adjuvant in the IL-4-/- mice. Thus, our data disputes the idea that the adjuvant mechanism of CT is dependent on Th2 functions. Moreover, previous studies have suggested that immunogenicity and adjuvanticity of CT are closely related phenomena (4). It is interesting to note that CT was found to stimulate significant local and systemic anti-CT responses after oral immunization whereas no or poor responses to the accompanying KLH or OVA were observed. Thus, CT was immunogenic but did not augment mucosal responses to the unrelated proteins. Therefore, we propose that the IL-4-/- mice provide a unique model in which immunogenic and adjuvant properties of CT may be dissociated and further analyzed.

To reconcile the fact that CT did not promote an intestinal KLH or OVA response but was effective in stimulating a response against itself, we hypothesized that CT has access to an alternative pathway for induction of gut mucosal immune responses. This pathway is probably separate from the PP pathway since despite strong anti-CT IgA responses in the gut no germinal centers were seen in PP of IL-4-/- mice. One possibility is that CT gains access to the MLN and stimulates antigen-specific T and B cells in this tissue rather than in the PP. This notion is supported by our finding of germinal center formations in the MLN of IL-4-/- mice after oral immunization. Probably uptake and transport of CT rather than KLH or OVA to the alternative pathway may be explained on the basis of CT's strong immunogenic and immunomodulating properties (16, 20, 23). For example, the GM1-ganglioside receptor for CT is widely distributed on mammalian cells (2). Therefore, CT, which binds its receptor with high affinity, may be effectively taken up and processed/presented to T cells by any of the gut mucosal APC, including B cells, dendritic cells, macrophages, or even epithelial cells (1, 2). These APC may be stationary in the tissue or be migrating from the PP to the MLN, for example. This may explain the induction of gut mucosal responses to CT rather than KLH or OVA after oral immunization in IL-4-/- mice. Future studies will experimentally address this possibility. The hypothesized pathway must have generated T helper activity that resulted in isotype distribution of anti-CT responses that closely resembled those found for other antigens studied in IL-4-/- mice (21, 24). However, of note, no serum anti-CT IgM was demonstrable in the IL-4-/- mice. This perhaps may indicate that an alternative pathway for induction of mucosal immune responses was used, especially since serum anti-CT antibodies in IL-4-/- mice after parenteral immunizations equalled the wild-type levels of anti-CT IgM (our unpublished result). This lack in serum anti-CT IgM after oral immunization may be due to a direct effect of CT on B cell isotype differentiation in IL-4-/- mice. Previously, we found that CT greatly promotes B cell isotype switch-differentiation at the gene transcriptional level in an isotype unrestricted fashion (20). Conceivably, CT might have directly induced switching in IL-4-/- B cells from IgM to other isotypes in the alternative pathway for induction of mucosal anti-CT responses.

Previous studies have demonstrated that IgA B cell differentiation might be regulated by cytokines, including TGF-β, IL-5, and IL-6 (31-34). Recently, also IL-4 was shown to increase switching to IgA in murine B cell lines (13, 14). To our surprise we found similar or even higher total IgA levels in serum, secretions, and lamina propria of IL-4-/- mice as compared with wild-type mice. Thus, the ability of B cells to undergo switch differentiation from IgM to IgA does not seem to be impaired in IL-4-/- mice, suggesting that IL-4 or Th2 cytokines may play a less important role for IgA B cell differentiation than has previously been assumed (14, 31, 35-37). Therefore, the inability to respond to oral immunization with mucosal antibody production in IL-4-/- mice was not the result of a defective differentiation pathway for IgA B cells but rather the failure to induce specific T and B cells in the PP. The origin of the lamina propria IgA precursor cells is unclear since PP may not be the site for antigen-stimulation (Figs. 3 and 4) (1, 38). As an alternative

Figure 5. Light level micrographs demonstrating the presence of germinal centers in the mesenteric lymph nodes from both the wild-type and IL-4-/- mice 10 d after an oral immunization with OVA and CT-adjuvant (x80). Immunofluorescence labeling indicating the PNA binding cells (green) constituting a germinal center (arrows) in wild-type (A) and IL-4-/- (B) mice.

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source for IgA plasma cells in the lamina propria earlier studies have proposed resident B cells in the peritoneal cavity (39). A majority of these B cells were of the self-replenishing B-1 phenotype and it awaits to be investigated if the repertoire of intestinal IgA antibodies in IL-4-/- mice exhibit the restricted antigen-recognition pattern characteristic of peripheral B-1 cells (39). Alternatively, as the mucosal IgA anti-CT response seems to suggest, local stimulation with antigen of bone marrow–derived B cells may occur in the MLN or even in situ in the lamina propria (40). Further studies are required to resolve this question.

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