ARTICLES

Clustering of Colonic Lamina Propria CD4⁺ T Cells to Subepithelial Dendritic Cell Aggregates Precedes the Development of Colitis in a Murine Adoptive Transfer Model

Frank Leithäuser, Zlatko Trobonjaca, Peter Möller, and Jörg Reimann

Department of Pathology (FL, PM) and Department of Medical Microbiology and Immunology (ZT, JR), University of Ulm, Ulm, Germany

SUMMARY: Initial lesions in inflammatory bowel disease induced during the repopulation of immunodeficient RAG1^{-/-} mice with immunocompetent CD4⁺ T cells have not been previously described. In this transfer colitis model, we followed CD4⁺ T cell repopulation in the host by injecting autofluorescent CD4⁺ T cells from congenic, enhanced green fluorescent protein (eGFP)-transgenic mice. This allowed the direct, sensitive, and unambiguous histological detection of the repopulation of the intestinal tract, mesenteric lymph nodes, and spleen of the host with donor eGFP⁺ CD4⁺ T cells. We identified in RAG1^{-/-} mice intestinal dendritic cell (DC) aggregates under the basal crypt epithelium at the mucosa/submucosa junction from which F4/80⁺ macrophages were excluded. At Days 8 to 11 posttransfer (before colitis was manifest), CD4⁺ T cells clustered and proliferated in CD11c⁺ DC aggregates. T cell clustering was most pronounced in the cecum where histologically overt colitis became manifest 5 to 10 days later. Junctional DC aggregates were thus prevalent in the triggering phase of the disease. The data suggest that pathogenic T cell responses inducing inflammatory bowel disease are primed or restimulated in situ in junctional CD4⁺ T cell/DC aggregates. (*Lab Invest* 2001, 81:1339–1349).

Early events preceding the first histopathological lesions or clinical signs of inflammatory bowel disease (IBD) have not been identified. We used an established murine adoptive transfer model to identify early in situ events that precede the histopathological manifestation of colitis. In this system the adoptive transfer of CD4⁺ αβ T cells into congenic, severely immunodeficient BALB/c H-2^d mice (Aranda et al, 1997; Morrissey et al, 1993; Powrie et al, 1994a, 1994b; Rudolph et al, 1994) or C57BL/6 (B6) H-2^b mice (Annacker et al, 2000; Corazza et al, 1999; Simpson et al, 1998) induces a colitis. Deregulated mucosal Th1 T cell responses leading to exaggerated interferon (IFN)-γ and tumor necrosis factor α responses are a major factor in the pathogenesis of colitis developing in these models (reviewed in de Winter et al, 1999; Strober et al, 1998). In this adoptive transfer model, we used CD4⁺ T cells from enhanced

green fluorescent protein (eGFP)-transgenic donor mice that express a stable marker (eGFP). After transfer of eGFP⁺ CD4⁺ T cells into congenic RAG1^{-/-} recipients, we could follow the repopulation of the host with T cells and the emergence of lesions in the target organ. Sensitive and specific in situ detection of adoptively transferred CD4⁺ T cells was achieved by applying a recently developed protocol of tissue processing (Leithäuser et al, 2001).

CD11c⁺ dendritic cells (DC) are potent antigen-presenting cells (APC) for T cells (reviewed in Sallusto and Lanzavecchia, 1999; Steinman et al, 1997a, 1997b). DC are found in the intestinal mucosa (MacPherson et al, 1995; MacPherson and Liu, 2000), especially in Peyer's patches (PP), within the intestinal epithelial cell (IEC) layer, diffusely distributed throughout the lamina propria (LP) or close to the epithelium (occasionally extending processes through the basement membrane into the IEC layer). T cells may be primed by DC in PP (Iwasaki and Kelsall, 2000; Ruedl et al, 1996; Ruedl and Hubele, 1997), by antigen-bearing DC trafficking to mesenteric lymph nodes (mLN) (Huang et al, 2000; Liu and MacPherson, 1993; Liu et al, 1998), or by DC in the gut LP. It is not known where and how mucosal T cell responses that can trigger the development of colitis are initiated.

Received April 3, 2001.

This work was supported by grants from the IZKF/A7 (University of Ulm) to FL and JR and from the Deutsche Forschungsgemeinschaft (DFG Re549/9-1) to JR.

Address reprint requests to: Dr. Frank Leithäuser, Department of Pathology, University of Ulm, Albert Einstein Allee 11, 89081 Ulm, Germany. E-mail: frank.leithaeuser@medizin.uni-ulm.de

When naïve peripheral T cells are transferred into lymphopenic hosts, they undergo homeostasis-driven proliferation and transiently acquire a memory-like phenotype (Cho et al, 2000; Goldrath et al, 2000). This is observed in standard transfer colitis models in which low numbers (3×10^5 cells/mouse) of purified, peripheral CD4⁺ T cells are injected into severely immunodeficient, congenic RAG^{-/-} hosts. Hence, it is on the “background” of homeostasis-driven, diffuse T cell proliferation that we have to find evidence for specific CD4⁺ T cell priming that may be involved in the induction of colitis. We searched, at early stages before the manifestation of the disease, in the target organ (the colon) for organized structures that contain APC and transplanted T cells. The identification of such structures may help us to identify the emergence of pathogenic T cell responses in the gut mucosa in well-defined preclinical IBD models. We detected CD11c⁺ DC aggregates under the basal crypt epithelium at the mucosa/submucosa junction (junctional DC aggregates) in the small and large intestine of nontransplanted and transplanted RAG1^{-/-} mice. Junctional DC aggregates were repopulated at an early time point posttransplantation by donor CD4⁺ T cells before the impending IBD. This was most striking in areas where severe colitis emerged shortly thereafter. CD4⁺ T cells accumulated and proliferated within these DC aggregates. Our study thus defines CD4⁺ T cell accumulation in junctional DC aggregates as a triggering event preceding the histological manifestation of colitis.

Results

A Murine Adoptive Transfer Model for Studying Early Events in the Immunopathogenesis of IBD

We isolated functional, splenic CD4⁺ T cells expressing a stable eGFP^{hi} marker from eGFP-transgenic (tg) B6 mice (Fig. 1A). These green fluorescent CD4⁺ T cells were transferred into congenic RAG1^{-/-} B6 hosts

(3×10^5 cells/mouse), which developed clinical signs of IBD (inactivity, ruffled fur, diarrhea, rectal prolapse, and loss of body weight) within 3 weeks (Fig. 1B). This IBD was progressive and always lethal. We did not detect a difference in the course and manifestation of IBD induced by the transfer of splenic CD4⁺ T cells from either normal (non-tg), or eGFP-tg B6 donor mice into RAG1^{-/-} B6 hosts. Loss of body weight was not a reliable indicator of IBD severity because some transplanted mice died in the second or third week post-transfer with little clinical evidence of IBD but a histopathology of severe colitis. The course of IBD in B6 RAG1^{-/-} hosts induced by the transfer of CD4⁺ T cells was more aggressive and showed less interindividual variability than the IBD induced in severe combined immunodeficient (SCID) mice by transfer of BALB/c-derived CD4⁺ T cells, which we described previously (Claesson et al, 1999).

eGFP⁺ T cells were easily visualized by fluorescent microscopy in fixed cryosections. The bright green fluorescence permitted detection of individual eGFP⁺ T cells at low magnification and in tissues with high autofluorescence (eg, bone marrow). The fluorescent signals of these cells were specific because no cells with comparable fluorescence were detectable in non-transplanted RAG1^{-/-} B6 control mice. We used this system to study early stages in the induction of CD4⁺ T cell-dependent colitis. We describe three stages. In the first stage, we found evidence for the repopulation of the immunodeficient host with the transferred T cells but no evidence for an emerging histopathology of the gut. In the second stage, we detected discrete pathologic changes emerging in selected areas of the colon preceding clear manifestations of colitis. The third stage comprises the well-known features of an established colitis, which has been extensively documented (Bonhagen et al, 1998; Bregenholt et al, 1998; Leach et al, 1996). Our interest is in histopathological features that indicate the transition from the first to the second stage in this experimental colitis.

Figure 1.

A, Purified enhanced green fluorescent protein (eGFP)^{hi} CD4⁺ T cells from transgenic (tg) B6 donor mice used for the transfer into RAG^{-/-} hosts. RAG^{-/-} B6 mice transplanted with CD4⁺ T cells from either eGFP⁺-tg, or non-tg B6 donor animals develop inflammatory bowel disease (IBD). B, Mean loss of body weight ($\pm$ SEM) of 5 mice/group is shown.

Early CD4⁺ T Cell Repopulation of RAG^{-/-} Mice

Low numbers of eGFP⁺ T cells were first detectable 5 days posttransfer in the spleen and the mLN of transplanted RAG1^{-/-} mice. At this early time point posttransplantation, only a few T cells were diffusely scattered throughout the spleen and mLN (Fig. 2, A and B). Two to three days later, splenic T cells preferentially accumulated in periarteriolar sheets, with a few T cells diffusely scattered in the red pulp (data not shown). The infiltration of T cells in the mLN rapidly increased from Day 5 to Day 10 posttransplantation, was diffuse, and involved the complete node. Because of the diffuse infiltration pattern, a distinction of medullary and cortical regions of the mLNs was not possible. Neither follicle-like structures nor high endothelial venules were detectable in mLN (Fig. 2C). Transplanted T cells were virtually absent from primary lymphoid organs. T cells were rarely detected in marrow from femur or vertebrae (Fig. 2D). Although very small, the thymus can be identified in RAG1^{-/-} mice. All regions of the thymus were devoid of T cells (Fig. 2E).

Adoptively transferred CD4⁺ T cells repopulated the entire intestinal tract of transplanted RAG1^{-/-} mice. The small intestine showed only a few eGFP⁺ T cells

diffusely distributed in the lamina propria, which were first detected at Day 7 after transplantation and remained low thereafter (data not shown). T cell numbers increased to moderate density between Days 9 and 11 posttransplantation only in the terminal ileum (in immediate proximity to the ileocecal valve). All regions of the small intestine showed a normal histology during the 3-week observation period. CD4⁺ T cells thus repopulate spleen, mLN, and the small and large intestine LP of the immunodeficient host.

Changes Preceding the Histopathological Manifestation of Colitis

Early repopulation of the RAG1^{-/-} host did not show a preferential accumulation of T cells in the target organ of the disease, ie, the colon. The number of T cells infiltrating the colonic LP (cLP) was as low (Fig. 3A) as was the number of T cells in the small intestine LP. In the first 8 days posttransplantation, the colonic mucosa was histologically normal (Fig. 3D). Most CD4⁺ T cells were found in the mucosa. No T cells were found in the muscularis propria or the subserosa of the colon wall. The first evidence of impending IBD was a submucosal edema accompanied by hyperemia of venules and a striking congestion of lymphatic vessels

Figure 2.

eGFP⁺ CD4⁺ cells in lymphatic organs during early phase of colonization. A, In the spleen, eGFP⁺ cells are diffusely distributed during the early phase of colonization. B, The mesenteric lymph nodes show a diffuse infiltration of low density with eGFP⁺ cells. C, The lymph node parenchyma lacks follicular structures and a separation in medullary/cortical regions (hematoxylin and eosin [H&E] staining). Venules are lined by a flat endothelium (arrows). High endothelial venules are not detectable. D and E, eGFP⁺ cells (arrow) are scarce in the bone marrow (D) and absent from the thymus (E).

Figure 3.

Stages of CD4⁺ cell colonization and disease development in the large intestine. A, During the early phase of colonization, few eGFP⁺ T cells diffusely populate the lamina propria of the colon. D, H&E staining shows a normal histology of the large intestine. B, At Day 9 after transplantation, focal accumulations of eGFP⁺ T cells are apparent in some regions of the lamina propria. E, The lamina propria and the upper submucosa show a moderate influx of inflammatory cells, including a few neutrophils (H&E staining). In the submucosa, an edema with congestion of lymphatic vessels is prominent. C, The large intestine of established inflammatory bowel disease (IBD) at Day 21 shows a dense infiltration of the colonic mucosa by eGFP⁺ cells. The infiltrate is of a predominantly diffuse pattern and often involves the submucosa as well. F, H&E section of the colon illustrates histopathology of severe colitis with dense inflammatory infiltrate, mucosal hypertrophy, branching of the cryptal epithelium, and frequent crypt abscesses (*asterisk*). The inflammatory infiltrate consists of a high number of polymorphonuclear granulocytes. The epithelium shows a loss of polarity, increased nuclear/cytoplasm ratio, an enhanced basophilia, and a virtually complete loss of goblet cells.

particularly prominent in the cecal part of the colon (Fig. 3E). This coincided with the appearance of an inflammatory infiltrate in the LP containing mainly T cells. Between Day 8 and Day 11, the number of T cells in the large intestine markedly increased, and the diffuse pattern of the T cell infiltrations changed into a predominantly patchy pattern. Dense accumulations of eGFP⁺ T cells formed predominantly in the cecum and in the rectosigmoid (Fig. 3B). This represented a clustering of T cells to intestinal DC aggregates (see "Discussion"). Moderate numbers of neutrophils (identified by peroxidase stains of cryosections, data not shown) were also present, but eosinophils and mast cells (identified by Toluidine Blue staining) were rare or absent in the mucosa, although found in low numbers in the submucosa (data not shown). The crypt architecture was well preserved during this phase.

Histopathology of Established CD4⁺ T Cell-Dependent Colitis

Severe colitis was established in RAG1^{-/-} mice by Day 21 posttransplantation. The dense T cell infiltrate was predominantly diffuse and lacked the focal accumulations characteristic of the initial stage of IBD (compare Panels B and C, Fig. 3). The thickening of the hyperplastic colonic mucosa was prominent, but the edema in the submucosa had largely regressed (Fig. 3F). The inflammatory infiltrate was most severe in the mucosa but also extended into the submucosa in severely

diseased areas. It contained numerous neutrophils that occasionally migrated through the epithelial membrane into the lumen of the gut. At this advanced stage of IBD, there were some epithelial erosions, although ulcerations were rare. The elongated crypts showed a distorted architecture, with frequent glandular branching and a proliferating basophil epithelium at the lower two-thirds of the crypts accompanied by an almost complete lack of goblet cells. An increased number of mitotic cryptal IEC, the partial loss of IEC polarity, a shift toward a higher nucleus/cytoplasm ratio, and a coarsened chromatin pattern in IEC indicated enhanced epithelial cell proliferation. This was confirmed by an increased bromodeoxyuridine (BrdU) incorporation in a large number of colonocytes (data not shown). Crypt abscesses were frequent.

Clustering of CD4⁺ T Cells in Colonic Lamina Propria DC Aggregates in Emerging IBD

T cell stimulation depends on APC required for the specific and major histocompatibility complex (MHC)-restricted epitope presentation, as well as for the modulation of the polarized (ie, Th1 or Th2) effector phenotype of the stimulated T cells. Candidate APC in the colonic mucosa are IEC, mast cells, B cells, macrophages, and DC. IEC are unlikely APC because no T cell infiltration was seen in the epithelium in the early stage of IBD development, and only a few T cells migrated into the colon epithelium late in established

IBD (data not shown). Mast cells were largely absent from the mucosa (data not shown). B cells are absent from RAG1^{-/-} mice and the T cell transplant. Hence, only macrophages or DC are candidate professional APC that could stimulate mucosal T cell responses in early IBD.

In the large intestine of untransplanted RAG1^{-/-} mice and in CD4⁺ T cell-transplanted RAG1^{-/-} mice with emerging (but not yet fully developed) colitis, a few CD11c⁺ DC were found diffusely distributed in the cLP (Fig. 4A). Macrophages (identified by the F4/80 mAb) were found in the cLP and in the colonic submucosa (Fig. 4B).

Prominent were large, dense aggregates of intestinal DC at the mucosa/submucosa junction, directly under the epithelium at the base of the crypts of the small and large intestine of nontransplanted and transplanted RAG1^{-/-} mice (Fig. 4, C and H). The DC patches were found at the base of the lamina propria in the mucosa (Fig. 4, D and G) or in the submucosa immediately below the muscularis mucosae (Fig. 4F). The muscularis mucosae covering DC aggregates located within the submucosa was delicate and discontinuous, thus permitting an intimate contact of DC with the cryptal base. Some, but not all, CD11c⁺ DC within these junctional aggregates were also DEC205⁺ and/or MHC-II⁺ (data not shown). F4/80⁺ macrophages were excluded from all aggregates analyzed (Fig. 4, E and I). At Days 9 to 11 after transplantation, the subepithelial DC aggregates in the colon, and to a lesser degree in the small intestine, showed an influx of CD4⁺ T cells (Figs. 3B and 4H). This was demonstrated by anti-CD4/anti-CD11c double staining of colonic mucosa with initial inflammatory alterations obtained from transplanted RAG1^{-/-} mice at Day 11 posttransfer (Fig. 4H). Hence, a conspicuous colocalization of CD4⁺ T cells with DC aggregates, but not with macrophages (Fig. 4I), was obvious in early IBD.

Purified cLP CD4⁺ T cells from transplanted RAG1^{-/-} mice with IBD released IFN γ when restimulated *in vitro* with syngeneic DC pulsed with lysates from gut bacteria. This interaction was MHC-II-restricted (Fig. 5). These IBD-associated cLP CD4⁺ T cells did not respond to stimulation with lysate-pulsed macrophages grown from bone marrow with macrophage colony-stimulating factor (M-CSF, data not shown). DC can thus efficiently present gut-derived bacterial antigens to LP CD4⁺ T cells associated with IBD.

At Day 21 posttransplantation, colitis was fully developed and the density of DC within the cLP was markedly increased. The infiltration pattern of DC in the inflamed cLP in advanced IBD was predominantly diffuse (Fig. 4J). At this stage of the IBD, there was also an increased number of F4/80⁺ macrophages within the colonic lamina propria (Fig. 4K).

Proliferation of Cells within T Cell/DC Clusters in Early IBD

We injected transplanted RAG1^{-/-} mice with BrdU 1 hour before euthanasia to label proliferating cells *in*

vivo. Proliferating CD4⁺ T cells (labeled with a mAb against BrdU) were detected *in situ* within junctional DC aggregates (Fig. 6, A and B). Proliferating T cells were also found in the cLP and mLN, but there were only a very few proliferating CD4⁺ T cells in the spleen (data not shown).

Discussion

At Day 5 posttransfer, few CD4⁺ T cells were seen in spleen and mLN. The influx of T cells into mLN seems to take place exclusively through afferent lymphatics, because high endothelial venules (HEV; required for an influx of naïve, blood-borne T cells into the mLN) are not developed. T cell numbers in mLN were very low until Day 9 posttransfer and showed a diffuse distribution pattern. The first CD4⁺ T cells were detected in the intestinal LP of the transplanted hosts at Days 7 to 9 posttransfer. The transferred CD4⁺ T cells selectively migrated to gut and gut-associated structures but did not repopulate bone marrow, thymus, or peripheral LN (not associated with the gut). The data extend and confirm our previously published observation in the SCID model (Bonhagen et al, 1996, 1998; Bregenholt et al, 1998; Claesson et al, 1996, 1999; Reimann et al, 1993, 1995, Reimann and Rudolph, 1995; Rudolph et al, 1994, 1996).

Professional APC are present in the intestinal mucosa of nontransplanted and transplanted RAG1^{-/-} B6 mice. They include mast cells, macrophages, and DC, but not B cells. Only a few mast cells were found in the submucosa of the small and large intestine. Macrophages (identified by mAb F4/80) were abundant in the submucosa and diffusely distributed in the LP of the mucosa. DC (identified by anti-CD11c) were abundant in the LP. Intestinal DC were found to diffusely infiltrate the LP preferentially in subepithelial areas and to cluster in junctional DC aggregates but were not found in the gut epithelium beyond the basal membrane as demonstrated by double staining of CD11c and laminin (data not shown). Conspicuous were the large junctional DC aggregates in the basal LP and the adjacent submucosa of the intestinal wall that were in intimate contact with the basal crypt epithelium. DCs were CD11c⁺ MHC-II⁺ throughout the aggregates. In some, but not all, DC aggregates, DEC205 expression was detected in a subset of DCs located in the basal regions of the DC aggregates. F4/80⁺ macrophages were completely excluded from the DC aggregates. It is possible that DC aggregates represent PP anlagen (Adachi et al, 1998). This notion is supported by a recent study (Debard et al, 2001) reporting the CD11c⁺ DC accumulations in the small intestine of RAG1^{-/-} mice. These DC accumulations closely resembled PP because they harbored FDC-M1⁺ follicular dendritic cells and were covered by a follicle-associated epithelium containing alkaline phosphatase negative M cells.

The influx of T cells into the junctional DC aggregates preceded the histopathological manifestations of colitis in the target tissue by 4 to 8 days. A key finding was that the diffuse T cell infiltration became

Untransplanted Rag1^{-/-} CD4⁺→Rag1^{-/-}, day 11 CD4⁺→Rag1^{-/-}, day 21 **Figure 4.**

Professional antigen-presenting cells (APC) at different stages of disease development. A, CD11c⁺ dendritic cells (DC) are diffusely distributed in the intestinal lamina propria of untransplanted RAG1^{-/-} mice but largely absent from the deeper layers of the colon. B, Macrophages, identified by F4/80 mAb, are present in the lamina propria as well as the submucosa. D and H, Large accumulations of CD11c⁺ cells can be found at the basal region of the colonic mucosa and the adjacent submucosa of untransplanted RAG1^{-/-} mice (D) and mice at Day 11 after transplantation with CD4⁺ T cells (H). E and I, F4/80-positive macrophages are largely excluded from these structures. H and I, In transplanted animals, double staining of CD4 (green) and CD11c or F4/80 (red) demonstrates colocalization of CD4⁺ T cells with CD11c⁺ DC (H), but not with F4/80⁺ macrophages (I). C and G, H&E staining of mucosal DC aggregates (arrows) reveals a close contact to the overlying epithelium. F, When located in the submucosa, DC aggregates are juxtaposed to the adjacent crypts by thinning or gaps (arrows) of the muscularis mucosae. J and K, At fully developed colitis at Day 21 after transplantation, the number of CD11c⁺ DC (J) and F4/80⁺ macrophages (K) diffusely infiltrating the lamina propria has markedly increased.

Figure 5.

Detection of interferon (IFN)- γ release in cLP CD4⁺ T cell/DC cocultures. IFN- γ was detected in supernatants of 3-day cocultures of cLP responder CD4⁺ T cells with bacterial lysate-pulsed DC using conventional double-sandwich ELISA. Mean IFN- γ values ($\pm$ SEM) of triplicates are shown.

Figure 6.

A and B, Proliferation of CD4⁺ cells within dendritic cell aggregates. Double staining of BrdU (green) and CD4 (red) demonstrates a considerable proportion of proliferating CD4⁺ cells mostly located at the bottom of dendritic cell aggregates at the mucosa/submucosa junction.

patchy because of the colonization of junctional DC aggregates with donor T cells. This patchy T cell infiltration pattern was most pronounced in the cecum, although T cells were also detectable in DC aggregates at other sites of the intestine. A high rate of T cell proliferation was observed within DC aggregates, and a large fraction of CD4⁺ T cells displayed an activated phenotype. The prominent and early influx of T cells into the junctional DC aggregates in areas of the colon that later develop typical colitic lesions suggests that in situ T cell priming or restimulation may be a key event in the pathogenesis of colitis.

DC aggregates were not prominent in areas of severe colitis and thus appear to be transient structures. The reason for the loss of DC aggregates during

the progression of disease is not entirely clear. Possibly, the aggregates were concealed in the dense inflammatory infiltrate containing CD4⁺ T cells, polymorphonuclear granulocytes, and large numbers of CD11c⁺ DC (Fig. 4J).

We transferred 3×10^5 splenic CD4⁺ $\alpha\beta$ T cells/mouse, the large majority of which expressed the CD45RB^{hi} CD44^{lo} surface phenotype of naïve T cells. These CD4⁺ T cells expanded in the adoptive RAG1^{-/-} host and expressed exclusively the surface phenotype of mucosa-homing effector/memory CD4⁺ T cells. Hence, either a very small population of colitis-inducing CD4⁺ T cells present in nondiseased B6 donor mice (where this subset must be tightly controlled) is expanded in the RAG1^{-/-} host, or transferred naïve CD4⁺ T cells are primed in the adoptive host to gut-derived antigens and

develop an IBD-inducing phenotype. The small number of memory/effector cells transplanted and the diverse TCR V β repertoire of CD4⁺ T cell populations recovered from the inflamed cLP (Z Trobonjaca, unpublished data) suggest that CD4⁺ T cells are primed to gut-derived antigens in the adoptive host. The data in Figure 5 show that CD4⁺ T cells specifically react to gut-derived antigens presented by DC, confirming and extending our previous report (Brimnes et al, 2001). This leads to the question of where CD4⁺ T cells are primed in the adoptive host. Organized mucosa-associated lymphoid tissue (MALT) structures (eg, typical PP) are not detectable in RAG1^{-/-} mice. Typical follicles and cortex/medulla compartments are absent from mLN. There were 2 to 4 days between the first detectable influx of T cells into the mLN and the clustering of T cells in DC aggregates in the cLP. Although the clustering of T cells into colonic DC aggregates was always very dense, T cell infiltration of the mLN showed a diffuse and scattered pattern. The first evidence for T cell accumulation and proliferation was found in cecal LP DC patches, and colitic lesions appeared a few days later in the same area. The type or load of antigen in the cecum may be a potent stimulus that drives T cell priming in situ. Transferred naïve T cells may thus be primed either in mLNs of the transplanted RAG1^{-/-} mice (and subsequently home to junctional DC aggregates in the cLP for specific restimulation, effector function delivery, and/or memory development) or in junctional DC aggregates in the cLP.

The data presented here suggest the possibility that CD4⁺ T cell responses to gut-derived antigens are initiated in junctional DC aggregates in the colonic mucosa/submucosa. These structures are prominent during the initiation of the CD4⁺ T cell-dependent colitis. Thus, the possibility has to be considered that priming of pathogenic CD4⁺ T cells may take place in the cLP at a time of perfect intestinal healthiness.

Materials and Methods

Mice

C57BL/6J (normal B6) mice, C57BL/6J-*Rag1*^{tm1Mom} (RAG1^{-/-}) mice (Mombaerts et al, 1992), and eGFP-tg B6 mice (Ikawa et al, 1998, 1999; Kawakami et al, 1999; Okabe et al, 1997) were used. RAG1^{-/-} B6 mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). eGFP-tg B6 were kindly provided by Dr. M. Okabe (Osaka University Research Institute for Microbial Diseases, Osaka, Japan). Breeding colonies of these mice were established in the animal colony of Ulm University, Ulm, Germany. Mice were bred and kept under specific-pathogen-free (SPF) conditions in the animal facility of Ulm University. RAG1^{-/-} mice were transplanted at 6 to 12 weeks of age.

Isolation and Adoptive Transfer of eGFP^{bright} CD4⁺ Cells

CD4⁺ T cells were aseptically purified from spleen cells of eGFP-tg B6 mice depleted of CD8⁺ T cells by treatment with anti-CD8 antibody and low-toxicity rabbit complement (Cedarlane, Hornby, Ontario, Canada) following the manufacturer's instructions. CD4⁺ T cells

were further enriched to more than 98% purity by positive selection on magnetic cell separation columns (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany). CD4^{hi} eGFP^{hi} cells were separated by FACS. The purity of the positively separated CD4⁺ population was always greater than 98%. Into RAG1^{-/-} B6 mice, we injected intraperitoneally 3×10^5 CD4⁺/mouse. At biweekly intervals, the transplanted mice were weighed and their clinical condition was monitored.

Tissue Processing

Tissue specimens were obtained from the duodenum, jejunum, terminal ileum, cecum, ascending colon, transverse colon, descending colon, rectum, mesenteric and peripancreatic lymph nodes, spleen, thymus (only mice from Days 5–13 after transplantation), and bone marrow (vertebrae and femur). At least three samples/time point were taken from each of these tissues. In situ detection of eGFP⁺ cells was done according to a recently published protocol (Leithäuser et al, 2001). Samples were fixed in 4% buffered paraformaldehyde-solution (pH 7.2) for 16 hours at 8° C, snap-frozen in liquid nitrogen, and stored at -70° C. A corresponding specimen was always processed for routine histology by fixation in 4% formalin (pH 7.0) for 24 hours and paraffin embedding. For immunohistology, a third sample was snap-frozen in liquid nitrogen and stored at -70° C. Paraformaldehyde- and formalin-fixed bone tissue was decalcified in an EDTA solution (200 g/L, pH 7.2) at 37° C for 1 week and subsequently processed as described above. No unfixed bone tissue was used in this study.

Histological Staining

From paraffin blocks, 2- μ m sections were cut with a microtome. These were submitted to automatic hematoxylin-eosin (H&E) staining. From selected positions and time-points, additional stainings (periodic acid Schiff [PAS], Giemsa, Toluidine Blue) were performed according to established protocols. Endogenous peroxidase was visualized on cryosections by the substrate 3-amino-9-ethylcarbazole (0.1 mg/ml in 0.17 M sodium acetate, pH 5.2, plus 0.01% H₂O₂) and counterstaining in hematoxylin.

Immunofluorescent Staining and Detection of eGFP⁺ Cells

mAb used for immunohistology were anti-CD4 clone L3T4, (Pharmingen, Hamburg, Germany), anti-CD11c clone HL3 (Pharmingen), anti-F4/80 clone A3-1 (Biozol Diagnostika GmbH, Munich, Germany), anti-mouse dendritic cell DEC205 clone NLDC-145 (Biozol Diagnostika), anti-laminin clone 4C12.8 (Immunotech, Marseille, France), anti-MHC-II clone M5/114, and anti-BrdU clone 3D4 (Pharmingen). mAb against CD4, CD11c, and F4/80 were biotin-labeled, anti-BrdU mAb was FITC-conjugated, and anti-DEC205, anti-MHC-II, and anti-laminin mAb were unconjugated. For single immunofluorescent staining, biotin-labeled antibodies were detected using Cy3-conjugated streptavidin (Di-

anova, Hamburg, Germany). Unconjugated mAb were detected using a polyclonal Cy3-conjugated donkey anti-rat IgG F (ab')₂ fragment (Dianova). For double staining of laminin and CD11c⁺ or CD4⁺ cells, bound anti-laminin mAb was detected by a polyclonal, Cy3-conjugated goat anti-rat-IgG Fab fragment (Dianova), and bound anti-CD11c mAb and anti-CD4 mAb were revealed by dichlorotriazinylaminofluorescein (DTAF)-conjugated streptavidin (Dianova). Double staining of CD4⁺ and CD11c⁺ or F4/80⁺ cells was carried out with a polyclonal, Cy2-conjugated goat anti-rat-IgG Fab fragment (Dianova) to label bound anti-CD4 mAb and Cy3-conjugated streptavidin to detect mAb against CD11c and F4/80. For this specific application, a purified anti-CD4 mAb clone L3T4 (Pharmin-gen) was used.

Frozen tissue was cut into 2- μ m sections on a cryostat-microtome (Leica, Wetzlar, Germany), mounted on coated microscopic slides, and acetone-fixed for 2 minutes at room temperature. These cryosections were incubated with the appropriate antibodies at pre-tested concentrations for 1 hour, washed twice in PBS, and stained by labeled streptavidin or a secondary antibody. In negative controls, the primary antibody was omitted. All incubation steps were carried out at room temperature in PBS.

Immunofluorescent stainings and cryosections of paraformaldehyde-fixed tissue were counterstained with 0.3 μ g/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich GmbH, Deisenhofen, Germany), treated with 70% EtOH for 30 minutes, and embedded in Vectashield medium (Vector, Burlingame, California).

BrdU Labeling

Proliferating CD4⁺ T cells were detected in situ by in vivo BrdU labeling. At Days 5 to 13 after adoptive transfer, animals were intraperitoneally injected 1 hour before euthanasia with 1 mg/mouse BrdU (Sigma-Aldrich) dissolved in 0.2 ml PBS. Cryosections were stained with anti-CD4 mAb and Cy3-labeled streptavidin, fixed for 30 minutes in 70% ethanol, denatured in 2 M HCl, and neutralized in borate-buffer (0.1 M, pH 8.0) as described (Penit, 1988). BrdU-positive nuclei were detected by the FITC-conjugated mAb clone 3D4 (Pharmin-gen). Immediately after staining and embedding in Vectashield mounting medium, sections were evaluated by fluorescence microscopy.

Fluorescence Microscopy

Slides stained with immunofluorescent reagents and cryosections of paraformaldehyde-fixed tissue containing eGFP⁺ cells were examined under an Axioskop-Microscope (Zeiss, Jena, Germany) using a mercury-vapor light source and an appropriate filter set. Pictures were recorded by a video camera and processed on a computer using the ISIS3-software, Version 3.02 (Metasystems, Heidelberg, Germany).

Generation of DC from Bone Marrow

The in vitro generation of DC from murine bone marrow has been described (Inaba et al, 1992). Briefly, bone marrow cells (BMC) prepared from femurs were depleted of CD4⁺ CD8⁺ B220⁺ lymphocytes and MHC-class-II⁺ cells (antibodies; Miltenyi Biotec, Bergisch-Gladbach, Germany) by MACS-sorting. These BMC depleted of T cells, B cells, and maturing myeloid cells were cultured at a density of 10⁶ cells/ml (Nunc, Wiesbaden, Germany) in serum-free UltraCulture medium (BioWhittaker, Verviers, Belgium) supplemented with 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml Flt-3 ligand (FL) (PeproTech, Rocky Hill, New Jersey), 2 mM glutamine, and antibiotics. Cultures were incubated at 37° C in humidified air with 5% CO₂. On Day 3 and Day 5, cells were fed by medium exchange. DC were harvested at Day 8 of culture. DC were pulsed with bacterial lysates for 2 hours, washed extensively, and used as stimulator cells.

Detection of IFN- γ Release in cLP CD4⁺ T Cell/DC Cocultures

IFN- γ was detected in supernatants of 3-day cLP CD4⁺ T cell/DC cocultures by conventional double-sandwich ELISA. For detection and capture, the mAb R4-6A2 (Pharmin-gen) and biotinylated mAb XMGI.2 (Pharmin-gen) were used. Extinction was analyzed at 405/490 nm on a TECAN micro plate-ELISA-reader (TECAN, Crailsheim, Germany) using EasyWin software (TECAN).

Acknowledgement

The expert technical assistance of Ms. Beate Wotschke and Ms. Anja Müller is gratefully acknowledged. We thank Drs. Mogens Claesson and Jens Brimnes (University of Copenhagen, Denmark) for their help and constructive discussion with the bacterial lysate-pulsed DC cultures.

References

- Adachi S, Yoshida H, Honda K, Maki K, Saijo K, Ikuta K, Saito T, and Nishikawa SI (1998). Essential role of IL-7 receptor- α in the formation of Peyer's patch anlage. *Int Immunol* 10:1–6.
- Annacker O, Burlen-Defranoux O, Pimenta-Araujo R, Cumano A, and Bandeira A (2000). Regulatory CD4 T cells control the size of the peripheral activated/memory CD4 T cell compartment. *J Immunol* 164:3573–3580.
- Aranda R, Sydora BC, McAllister PL, Binder SW, Yang HY, Targan SR, and Kronenberg M (1997). Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4⁺ CD45RB^{hi} T cells to SCID recipients. *J Immunol* 158:3464–3473.
- Bonhagen K, Thoma S, Bland P, Bregenholt S, Rudolphi A, Claesson MH, and Reimann J (1996). Cytotoxic reactivity of gut lamina propria CD4⁺ $\alpha\beta$ T cells in SCID mice with colitis. *Eur J Immunol* 26:3074–3083.
- Bonhagen K, Thoma S, Leithäuser F, Möller P, and Reimann J (1998). A pancolitis resembling human ulcerative colitis

(UC) is induced by CD4⁺ TCR $\alpha\beta$ T cells of athymic origin in histocompatible severe combined immunodeficient (SCID) mice. *Clin Exp Immunol* 112:443–452.

Bregenholt S, Reimann J, and Claesson MH (1998). Proliferation and apoptosis in lamina propria CD4⁺ T cells from scid mice with inflammatory bowel disease. *Eur J Immunol* 28:3655–3663.

Brimnes J, Reimann J, Nissen MH, and Claesson MH (2001). Enteric bacterial antigens activate CD4⁺ T cells from *scid* mice with inflammatory bowel disease. *Eur J Immunol* 31:23–31.

Cho BK, Rao VP, Ge Q, Eisen HN, and Chen J (2000). Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J Exp Med* 192:549–556.

Claesson MH, Bregenholt S, Bonhagen K, Thoma S, Möller P, Grusby MJ, Leithäuser F, Nissen MH, and Reimann J (1999). Colitis-inducing potency of CD4⁺ T cells in immunodeficient, adoptive hosts depends on their state of activation, IL-12 responsiveness, and CD45RB surface phenotype. *J Immunol* 162:3702–3710.

Claesson MH, Rudolphi A, Kofoed S, Poulsen SS, and Reimann J (1996). CD4⁺ T lymphocytes injected into SCID mice lead to an inflammatory and lethal bowel disease. *Clin Exp Immunol* 104:491–498.

Corazza N, Eichenberger S, Eugster H-P, and Mueller C (1999). Nonlymphocyte-derived tumor necrosis factor is required for induction of colitis in recombinant activating gene (RAG)2^{-/-} mice upon transfer of CD4⁺ CD45RB^{hi} T cells. *J Exp Med* 190:1479–1491.

Debard N, Sierro F, Browning J, and Kraehenbühl JP (2001). Effect of mature lymphocytes and lymphotoxin on the development of the follicle-epithelium and M cells in mouse Peyer's patches. *Gastroenterology* 120:1173–1182.

de Winter H, Cheroutre H, and Kronenberg M (1999). Mucosal immunity and inflammation. II. The yin and yang of T cells in intestinal inflammation: Pathogenic and protective roles in a mouse colitis model. *Am J Physiol* 276:G1317–G1321.

Goldrath AW, Bogatzki LY, and Bevan MJ (2000). Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J Exp Med* 192:557–564.

Huang FP, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, and MacPherson GG (2000). A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med* 191:435–444.

Ikawa M, Yamada S, Nakanishi T, and Okabe M (1998). "Green mice" and their potential usage in biological research. *FEBS Lett* 430:83–87.

Ikawa M, Yamada S, Nakanishi T, and Okabe M (1999). Green fluorescent protein (GFP) as a vital marker in mammals. *Curr Top Dev Biol* 44:1–20.

Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, and Steinman RM (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693–1702.

Iwasaki A and Kelsall BL (2000). Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3 α , MIP-3 β , and secondary lymphoid organ chemokine. *J Exp Med* 191:1381–1394.

Kawakami N, Sakane N, Nishizawa F, Iwao M, Fukada SI, Tsujikawa K, Kohama Y, Ikawa M, Okabe M, and Yamamoto H (1999). Green fluorescent protein-transgenic mice: immune functions and their application to studies of lymphocyte development. *Immunol Lett* 70:165–171.

Leach MW, Bean AG, Mauze S, Coffman RL, and Powrie F (1996). Inflammatory bowel disease in C. B-17 SCID mice reconstituted with the CD45RB^{high} subset of CD4⁺ T cells. *Am J Pathol* 148:1503–1515.

Leithäuser F, Trobonjaca Z, Reimann J, and Möller P (2001). In situ characterization of genetically targeted (green fluorescent) single cells and their microenvironment in an adoptive host. *Am J Pathol* 158:1975–1983.

Liu L, Zhang M, Jenkins C, and MacPherson GG (1998). Dendritic cell heterogeneity in vivo: Two functionally different dendritic cell populations in rat intestinal lymph can be distinguished by CD4 expression. *J Immunol* 161:1146–1155.

Liu LM and MacPherson GG (1993). Antigen acquisition by dendritic cells: Intestinal dendritic cells acquire antigen administered orally and can prime naive T cells in vivo. *J Exp Med* 177:1299–1307.

MacPherson GG, Jenkins CD, Stein MJ, and Edwards C (1995). Endotoxin-mediated dendritic cell release from the intestine. Characterization of released dendritic cells and TNF dependence. *J Immunol* 154:1317–1322.

MacPherson GG and Liu L (2000). Intestinal dendritic cells. In: Lotze MT and Thomson AW, editors, *Dendritic cells*. San Diego: Academic Press, 142–151.

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, and Papaioannou VE (1992). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869–877.

Morrissey PJ, Charrier K, Braddy S, Liggitt D, and Watson JD (1993). CD4⁺ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4⁺ T cells. *J Exp Med* 178:237–244.

Okabe M, Ikawa M, Kominami K, Nakanishi T, and Nishimune Y (1997). "Green mice" as a source of ubiquitous green cells. *FEBS Lett* 407:313–319.

Penit C (1988). Localization and phenotype of cycling and post-cycling murine thymocytes studied by simultaneous detection of bromodeoxyuridine and surface antigens. *J Histochem Cytochem* 36:473–478.

Powrie F, Correa Oliveira R, Mauze S, and Coffman RL (1994a). Regulatory interactions between CD45RB^{high} and CD45RB^{low} CD4⁺ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* 179:589–600.

Powrie F, Leach MW, Mauze S, Menon S, Barcomb-Caddle L, and Coffman RL (1994b). Inhibition of Th1 responses prevents inflammatory bowel disease in SCID mice reconstituted with CD45RB^{hi} T cells. *Immunity* 1:553–562.

Reimann J and Rudolphi A (1995). Coexpression of CD8 α in peripheral CD4⁺ TCR $\alpha\beta$ ⁺ T cells migrating into the murine small intestine epithelial layer. *Eur J Immunol* 25:1580–1588.

Reimann J, Rudolphi A, Spiess S, and Claesson MH (1995). A gut-homing, oligoclonal CD4⁺ T cell population in severe-combined immunodeficient mice expressing a rearranged, transgenic class I-restricted $\alpha\beta$ T cell receptor. *Eur J Immunol* 25:1643–1653.

- Reimann J, Rudolphi A, Tscherning T, and Claesson MH (1993). Selective engraftment of memory CD4⁺ T cells with an unusual recirculation pattern and a diverse T cell receptor-V β repertoire into SCID mice. *Eur J Immunol* 23:350–356.
- Rudolphi A, Boll G, Poulsen SS, Claesson MH, and Reimann J (1994). Gut-homing CD4⁺ TCR $\alpha\beta$ ⁺ T cells in the pathogenesis of murine inflammatory bowel disease. *Eur J Immunol* 24:2803–2812.
- Rudolphi A, Bonhagen K, and Reimann J (1996). Polyclonal expansion of adoptively transferred CD4⁺ $\alpha\beta$ T cells in the colonic lamina propria of SCID mice with colitis. *Eur J Immunol* 26:1156–1163.
- Ruedl C and Hubele S (1997). Maturation of Peyer's patch dendritic cells in vitro upon stimulation via cytokines or CD40 triggering. *Eur J Immunol* 27:1325–1330.
- Ruedl C, Rieser C, Böck G, Wick MJ, and Wolf H (1996). Phenotypic and functional characterization of CD11c⁺ dendritic cell population in mouse Peyer's patches. *Eur J Immunol* 26:1801–1806.
- Sallusto F and Lanzavecchia A (1999). Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. *J Exp Med* 189:611–614.
- Simpson SJ, Shah S, Comiskey M, De Jong YP, Wang B, Mizoguchi E, Bhan AK, and Terhorst C (1998). T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin 12/signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon gamma expression by T cells. *J Exp Med* 187:1225–1234.
- Steinman RM, Pack M, and Inaba K (1997a). Dendritic cell development and maturation. *Adv Exp Med Biol* 417:1–6.
- Steinman RM, Pack M, and Inaba K (1997b). Dendritic cells in the T-cell areas of lymphoid organs. *Immunol Rev* 156:25–37.
- Strober W, Ludviksson BR, and Fuss IJ (1998). The pathogenesis of mucosal inflammation in murine models of inflammatory bowel disease and Crohn disease. *Ann Intern Med* 128:848–856.