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The Roles of Invariant NKT Cells in Bowel Immunity — Suppression of Tumor Progression and Rejection of Intestinal Transplants

Tatsuaki Tsuruyama and Wulamujiang Aini

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1. Introduction

The roles of natural killer (NK)T cells in intestinal immunity have not been sufficiently investigated. The bowel possesses its own unique mucosal immune system, the gut-associated lymphoepithelial tract (GALT). In this review, we focused on CD1d-independent invariant type NKT (iNKT) cells as modulators of GALT. iNKT cells have a restricted invariant T-cell receptor (TCR) V α 24 chain paired with V β 11 chain. In addition to T helper (Th)1 cytokines, such as IFN- γ , iNKT cells can produce anti-inflammatory Th2 cytokines, such as interleukin (IL)-4.

Although invariant NKT cells are rare in normal small-intestine mucosa, they have been observed in intestinal allografts during rejection. Infiltrating iNKT cells release IL-4 and IL-5, Th2-related cytokines that antagonize the Th1 responses that induce acute cellular rejection. Also, iNKT cells form an immunological barrier against parasite infection. We found that CD1d + cells are actually localized in the lamina propria of the villi in the human intestine and they may present the antigen for the recruited iNKT cells.

On the other hand, a small number of invariant NKT cells are resident in the normal colorectal mucosa. iNKT cells are involved in defense against colorectal tumor progression and metastasis through the apoptosis-inducing molecule Fas ligand (FasL). Their numbers increase markedly in colorectal carcinomas. Increased iNKT cell infiltration in colorectal carcinomas is an independent favorable prognostic factor. iNKT cells probably play an important role in the pathogenesis of ulcerative colitis.

Thus, iNKT cells are modulators of bowel mucosal immunity.

2. Background

2.1. Gut-associated lymphoepithelial tract (GALT)

The bowel possesses a distinct mucosal immune system, known as the gut-associated lymphoepithelial tract (GALT), which is comprised of high endothelial venules (HEVs) located in the inter-follicular region (IFR) around the Peyer's patches (PPs). GALT-associated immunity involves an exquisite balance between activation and suppression mediated through the release of cytokines [1] (Figure 1). The GALT contains the largest collection of lymphocytes in the body. Physiologic and barrier functions of GALT against bacterial infection are associated with mucosal surfaces and are essential; therefore, only limited damage can be tolerated. The mucosa is continuously exposed to a vast array of antigens, necessitating the distinguishing of self from non-self/infectious antigens. Infectious agents, malignant tumors such as adenocarcinomas, and immune and inflammatory bowel diseases of the gut all contribute to morbidity and mortality.

The lymphocytes in the bowel are believed to play pivotal roles in bowel immunity. Two types of lymphocytes are found in the bowel: intra-epithelial lymphocytes (IELs) and lamina propria (LP) lymphocytes (LPLs). In addition, the PPs are comprised of B cells and T cells, unique lymphocyte subsets that interact with M epithelial cells. Antigens are taken up by the PPs through the M-cell layer, inducing an immune reaction (Figure 1).

In addition, lymphocytes from the GALT circulate in the common mucosal immune system, including the mesenteric lymph nodes.

LPLs play important roles in immune homeostasis. Although these cells function in mucosal defense against pathogens, they ignore non-pathogenic bacteria and foods, a phenomenon known as oral tolerance. LPLs also play a supporting role in barrier defenses and are involved in the pathogenesis of inflammatory intestinal diseases. At the onset of Crohn's disease, mucosal T cells appear to mount a typical T helper (Th)1 response that resembles an acute infectious process, but this response is lost with progression to later stages of Crohn's disease. Such polarization of bowel cytokines is believed to be responsible for inflammatory bowel diseases (IBD).

2.2. Natural killer T cells

NKT cells are associated with both innate and adaptive immunity due to their ability to interact with a broad spectrum of antigens.

NKT cells are currently classified into three types classical NKT cells (non-classical NKT cells); and NKT-like cells, or CD1d-independent NK1.1+T cells [2]. Classical NKT cells express CD161 [2, 3]. Human classical NKT cells express an invariant TCR, V α 24, whereas mouse NKT cells express TCRV α 14 [4, 5]. In contrast, the T-cell receptor V regions expressed by non-classical NKT cells and NKT-like cells are highly diverse. CD56-NKT cells are believed to function as effector or regulatory cells in innate immunity.

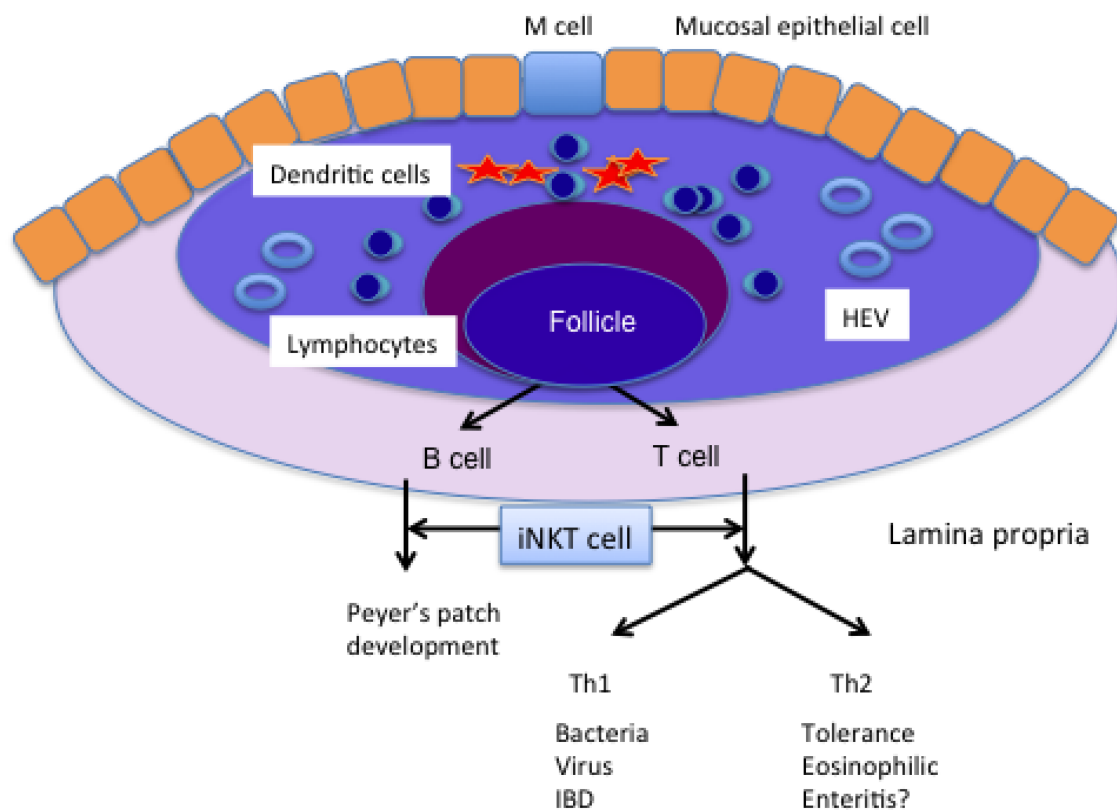


Figure 1. GALT and iNKT cells. M cells and mucosal epithelial cells cover the lamina propria of the bowel. HEV: high endothelial venules, Th1 & Th2, helper T cell Type 1 & 2. iNKT cells relate to the development of B cells and T cells in the lamina propria by release of cytokines such as IL-4, IL-5, IFN- γ .

2.3. Invariant natural killer T (iNKT) cells

Among these NK cells, iNKT cells have a very restricted TCR repertoire, which in human peripheral blood consists of an invariant V α 24-J α 18 chain (formerly V α 24-J α Q) paired with a V β 11 chain [4, 6]. Therefore, this classical type is termed “invariant” NKT (iNKT) cells (Figure 2). Human iNKT cells can be activated by glycolipid antigens such as beta-galactosylceramide and iGb3, which are presented by CD1d expressed on dendritic cells [7]. When activated, iNKT cells immediately produce pro-inflammatory Th1 cytokines such as IFN- γ and tumor necrosis factor (TNF)- α as well as anti-inflammatory Th2 cytokines, such as IL-4, IL-10, and IL-13. Thus, iNKT cells are capable of bidirectional differentiation, which is probably directed by the immunological environment.

iNKT cells are localized primarily in the thymus, bone marrow, liver, and spleen, and they are rare in the lymph nodes [8]. Bannai et al. [9] reported that mouse colon contains a considerable number of NKT cells. More recently, O’Keeffe et al. reported that T cells expressing CD161+(a phenotypic marker of NK cells) are the major natural killer receptor-positive cell population in the intestine. Although the intestines harbor a diverse array of NKT cells, iNKT cells are rare in the small intestine [10].

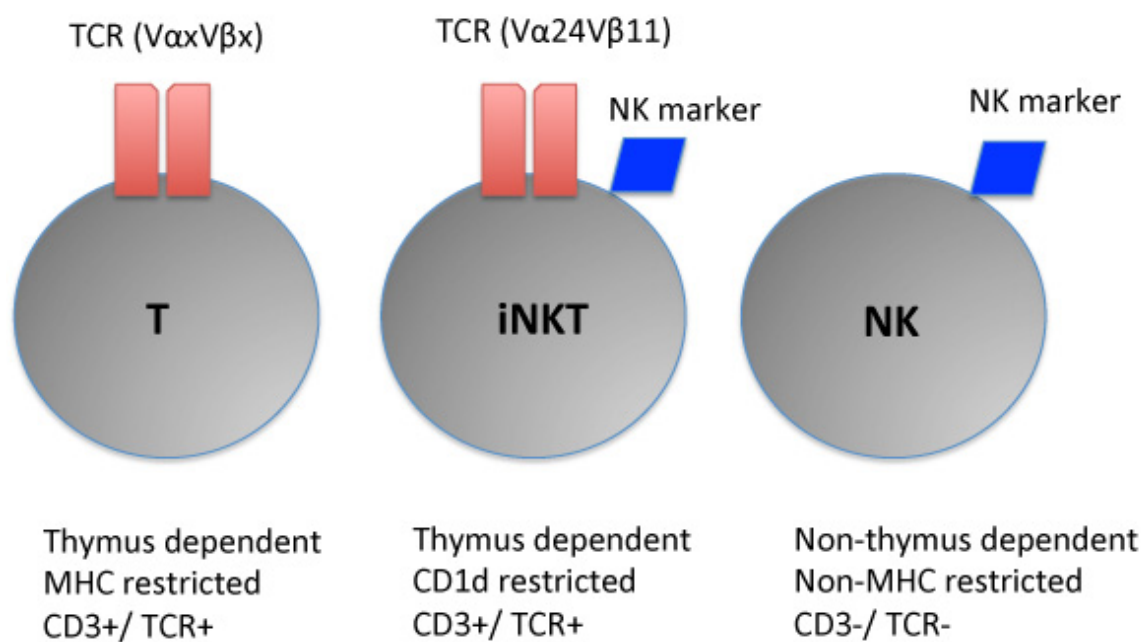


Figure 2. T, invariant natural killer (NK)T, and NK cells. The phenotypic surface antigens are shown. The T cell receptor (TCR) is variable in T cells; the TCR on the invariant type of human NKT cells is restricted to the Vα24β11 type. NK cells do not carry a TCR and express CD161, CD56, or CD57 NK markers, which are also expressed by NKT cells.

2.4. Invariant natural killer T (iNKT) cells in diseases

iNKT cells have been implicated as playing roles in infection control [11, 12], inflammatory bowel diseases [13], abortion [14], transplantation tolerance [15, 16], suppression of autoimmune diseases [17-20], psoriasis and atopic dermatitis [21], and the regulation of allergic disorders [22, 23]. iNKT cells have the potential to activate or suppresses immune system by release of variable cytokines including IFN-γ, IL-4, IL-10, and IL-17.

Moreover, a number of reports have suggested that iNKT cells play protective as well as tolerogenic roles in tumor immunity [24-29]. In addition, infiltration of iNKT cells is a prognostic indicator for colorectal cancer metastasis [29].

3. iNKT cells in the small intestine

3.1. Mucosal immunity of the small intestine

The mucosal immune system of the small intestine is unique. Mucosal barrier damage and inflammation have been associated with high levels of flagellin protein in the lumen. One of the roles of intestinal immunity is defense against pathogens within the bacterial flora. The mucosa is covered by mucin, which is one of the central determinants of gut immune specificity and immune tolerance.

The LP, located beneath the mucosa, includes various types of immune cells that form the GALT. The PP is a predominant immune apparatus that is covered by M cells and is capable of controlling mucosal immunity. In addition to the lymph apparatus and lymphocytes, the intestinal immune system contains dendritic cells (DCs) that interact with goblet cells. Mucus sensing enhances DC tolerogenic functions, including the release of TGF- β and suppressive IL-10, which are factors that induce FoxP3+regulatory T (T_{reg}) cell production. Populations of CD3+TCR- $\gamma\delta$ cells are also observed [29]. CD56+NK cells are classically viewed as lymphocytes that provide innate immunity against virus-infected cells and tumor cells through the release of cytolytic mediators and IFN- γ .

3.2. iNKT cells in the small intestine and coeliac disease

Intestinal iNKT cells are rare in the normal status [10], but small population is observed in the villi of the small intestine [30]. Although they have not been considered as minor participants in the control of GALT. However, iNKT cells are found to decrease in the celiac disease without affecting development of classical T cells. Coeliac disease is one of the autoimmune disorders of the small intestine. This disease occurs in genetically predisposed people of all ages from middle infancy. Symptoms include pain, chronic constipation and diarrhea in children. Diagnoses are occasionally being made in asymptomatic persons in the screening examination. Importantly, coeliac disease is not allergic disease, but caused by a reaction to a gluten protein in wheat. When the small intestine is exposed to gluten, the tissue transglutaminase modifies the protein, and the GALT autoimmunologically cross-reacts with the small intestinal tissue, causing an inflammatory reaction. As a result, villous atrophy is observed histologically and endoscopically. The absorption of nutrients is significantly affected. The effective treatment is a lifelong gluten-free diet. The deficiency of V α 24+ cells in coeliac disease was independent of age, gluten status of diet or duration of gluten-free diet. Probably, T cells are selected by glycolipids on CD1d expressing CD4+CD8+thymocytes [30].

3.3. Small-intestine allograft immunity

3.3.1. Background on intestinal transplantation

Intestinal transplantation is a commonly accepted standard therapy for patients with irreversible parenteral nutrition complications associated with short-bowel syndrome, [31-33], Hirschsprung and related diseases, chronic idiopathic intestinal pseudo-obstruction syndrome, and megacystis-microcolon-intestinal hypoperistalsis syndrome [34, 35]. Acute cellular rejection (ACR) remains the major cause of intestinal graft failure after transplantation. Diagnosis of ACR requires detection by histopathologic evaluation [35-38] as well as clinical examination and laboratory data [38].

3.3.2. Histologic criteria for diagnosis of ACR of small-intestine transplants

Pathologists have diagnosed acute rejection based on the finding of crypt apoptosis in the intestinal mucosa upon histologic examination of the graft [36-38], rather than lymphocytic

infiltrates. The number of apoptotic bodies is certainly augmented during the acute rejection; however, the mechanism underlying this phenomenon has not been determined.

Mucosal damage cannot be prevented once crypt apoptosis has become evident [38]. It is possible that apoptosis of crypt cells is induced by direct attack by cytotoxic T-lymphocytes (CTLs), but such a finding has not been reported. Therefore, it remains unclear whether CTL infiltration is necessary for ACR. Humoral factors may also play a role in crypt apoptosis.

Although there are reports describing T-cell apoptosis in the intestine in ongoing ACR, its clinical significance has not been determined [39]. T cells interact with Fas on other T cells to induce their apoptosis [40]. The apoptotic T cells are then phagocytosed by macrophages in the small-intestine allografts. Similar findings in a liver allograft has been reported by us [41]. Thus, these phagocytic findings may be common to various allografts in ACR. The phagocytosing macrophages form granulomas. Furthermore, apoptotic bodies are also positive for Fas, which is a surface antigen of activated T lymphocytes. Treatment with a steroid pulse significantly diminishes the apoptotic and phagocytotic responses that are indicative of ACR [42]. Although it is possible that both recipient and donor lymphocytes interact with each other via Fas and FasL molecules to induce apoptosis, a study using animal models reported that TNF- α (rather than interaction between Fas and FasL) is the primary factor for induction of lymphocytic apoptosis [39].

3.3.3. *iNKT cells infiltrate during acute rejection of the allograft*

Using systematic immunohistochemical analysis of an intestinal allograft, we assessed the behavior of iNKT cells during acute rejection of the intestinal allograft [38]. In practice, the diagnosis of intestinal rejection is difficult, particularly due to the complicated interactions between lymphocytes and other immunological cells resulting from the transient coexistence of donor-derived and recipient-derived cells in the graft.

iNKT cells are transiently recruited to the intestinal LP (Figure 3), but immunosuppressive therapy significantly decreased iNKT cells in number (Figure 4&5). The infiltrating intestinal iNKT cells have the potential to produce IL-4, which antagonizes IFN- γ and contributes to the development of Th2 cells. Higher levels of IL-4 prior to and shortly after kidney transplantation have been reported, and IL-4 may have a protective effect on renal graft survival [43]. Indeed, iNKT cells have been implicated in tolerance in experimental mouse models, in induction of chimerism in allogeneic cardiac transplant models [44], and in acceptance of rat-islet xenografts in mice [16]. IFN- γ production remains low relative to normal donor intestine and does not change during the course of ACR development. Therefore, infiltration of iNKT cells implies that they are involved in the response to rejection. Asaoka et al. reported the activation of CTLs in granzyme B/perforin-mediated graft injury [45]. Therefore, release of Th2-related cytokines by iNKT cells may antagonize this activation.

3.3.4. *PPs and iNKT cells in small-intestine allografts*

After transplantation, recipient-derived T cells traffic into the allograft across the HEVs located in the IFR around the PPs. Therefore, the PP serves as the interface through which the allograft

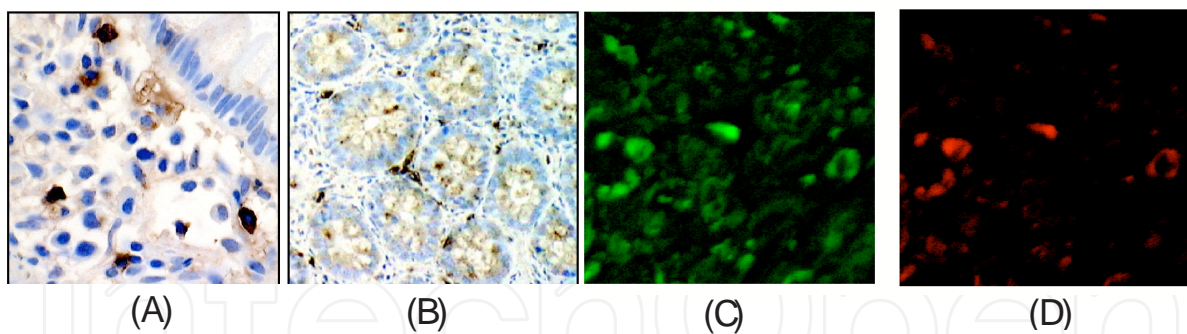


Figure 3. Immunohistochemistry of iNKT cells: (A) TCRVa24 (left) and (B) TCRVβ11 stained cells in brown. Fluorescent immunostaining of TCRVa24 (C), IL-4 (D).

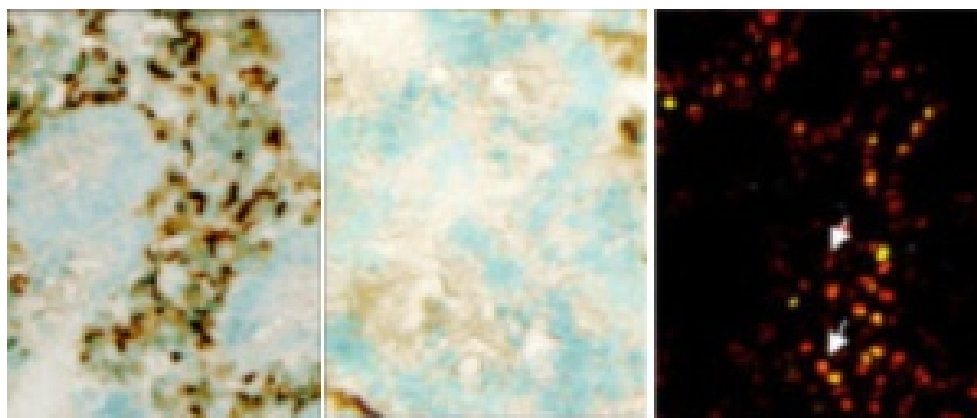


Figure 4. Time-course analyses using allograft tissues showed a significant decrease in the number of iNKT cells after immunosuppressant administration. The left photo illustrates the significant decrease in the number of iNKT cells after administration. Immunostaining of iNKT cells at the onset of ACR (left) and 48 hrs after administration of steroids (middle) is shown. Brown cells are iNKT cells in the allograft mucosa. Counterstaining was performed using methyl-green. iNKT cells underwent apoptosis in the graft. The right photo below shows TUNEL-positive (yellow) iNKT cells. Non-iNKT cells were stained red (phycoerythrin-labeled). This photo is the serial section of the left section [39].

encounters recipient-derived cells. Although, few studies have examined injury to the PPs during rejection, histology of PP showed the hyperplastic change of PP with an increase in expression of CD20, a mature B cell marker at the onset of rejection (Figure 6).

PPs consist primarily of B cells, T cells and DCs. In general, B cells carry out a variety of immune functions, including immunoglobulin production, cytokine release, antigen presentation, regulation of DC activity, and participation in the induction of immune tolerance [46]. A large number of T cells and B cells are present in the intestinal LP. The majority of T cells in the LP express the TCRαβ, and the proportion of CD8+T cells to CD4+T cells ranges from 2:1 to 3:1. The production of IL-4 and IL-5 by CD4+T cells in the LP promotes IgA production in the intestinal tract. Furthermore, CD8+T cells in the LP are responsible for damage by recognizing and activating macrophages. The primary antibodies secreted into the intestinal tract mucus are IgA. A number of IgA-producing cells distribute in the LP, and IgA molecules that are secreted into the intestinal tract are transported to the gut luminal side by binding to multimeric antibody receptors that are retained on the intestinal epithelial cells.

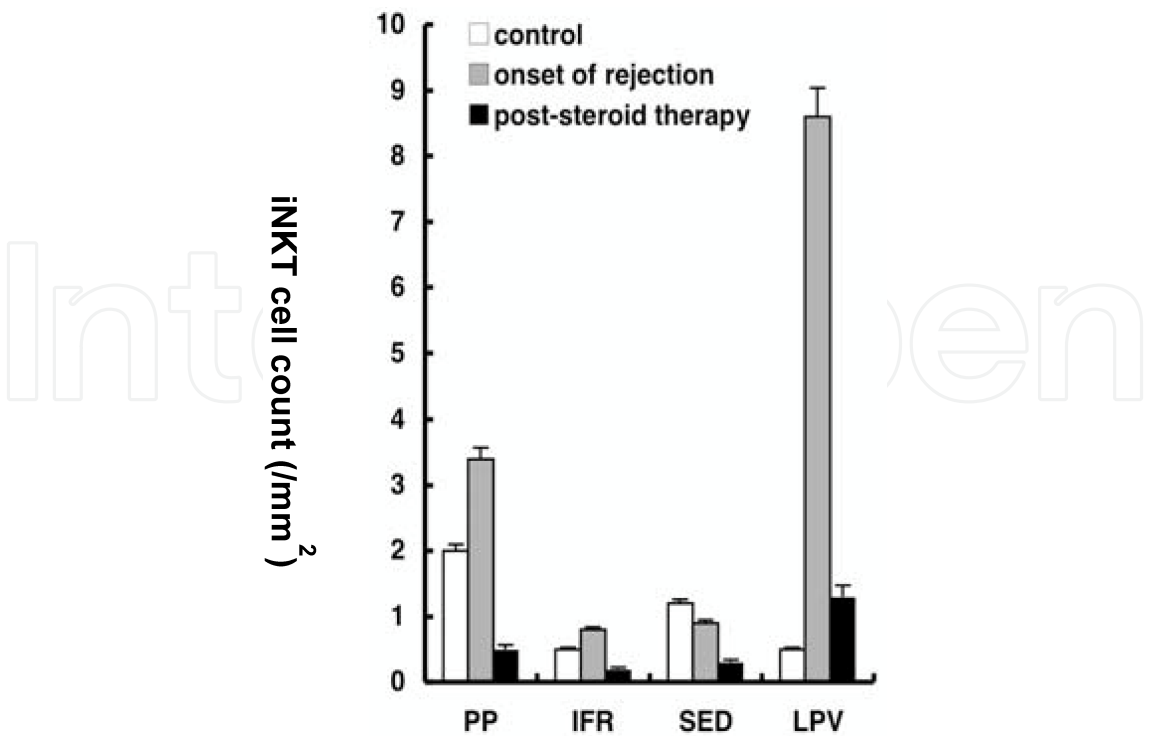


Figure 5. Distribution of iNKT cells in six intestinal allografts. The count of iNKT cells was evaluated by their number per 1mm² of a section. PP, Peyer’s patch; IFR, inter-follicular region; SED, mucosal sub-epithelial dome; LPV, lamina propria of the villi. Individual bars of intact, onset, and post-administration display the TCRVα24+T cell counts in the intact mucosa, in the mucosa with rejection, and in the mucosa 48 hours after >30mg of steroid administration (post-steroid therapy).

Both B-1 and B-2 cells produce IgA. B-2 cells are derived from PP B cells and exhibit a CD23+, CD11b-, CD5-, and CD19^{low} phenotype. Follicular B cells in the PPs are stimulated by antigen in the intestinal lumen and differentiate from IgM+ to IgA+ cells by class switching. IgA+ cells in the PPs circulate throughout the body via the thoracic duct and differentiate into IgA-producing cells by the effect of IL-6 produced by intestinal epithelial cells. B-1 cells, in contrast, are derived from B cells of the abdominal cavity and exhibit a CD19+, CD23-, and CD11b+ (B-1a, CD5+; B-1b, CD5-) phenotype. Whereas B-2 cell-derived secretory IgA recognizes foreign antigens with high affinity, B-1 cell-derived secretory IgA recognizes common bacterial phosphorylcholine, lipopolysaccharide, and self-antigens with low affinity.

3.3.5. Cytokine production in the intestinal allograft

Cytokine measurements have suggested that the levels of a variety of cytokines are increased at the onset of ACR and that TNF-α contributes to mucosal damage in graft-versus-host disease [47]. The graph below shows the production of various cytokines in graft mucosal tissues without PPs before transplantation, during ACR, and 72 hrs after administration of a steroid pulse. The data show that production of both IL-4 and IL-5 increase significantly relative to other cytokines in the allograft tissue during ACR, suggesting that ACR involves hypercytokinemia (Figure 7)[38].

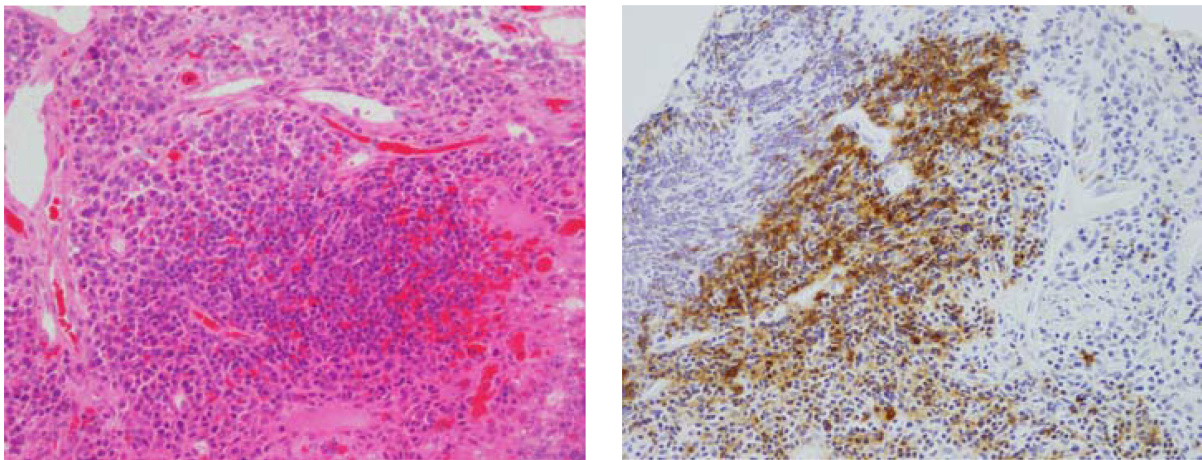


Figure 6. Peyer's patch (PP) hyperplasia in acute rejection of small intestinal allograft. Left photo shows an H&E stained hyperplastic PP in the graft at the onset of rejection. Right photo shows the CD20+ stained hyperplastic PP.

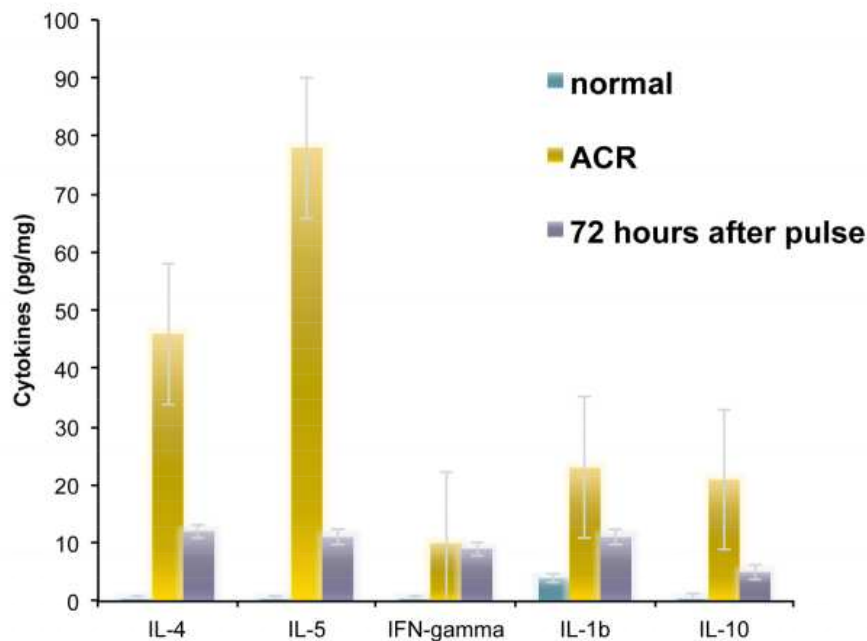


Figure 7. Cytokine concentration in the histologic normal allograft, at the onset of acute cellular rejection (ACR) and 72 hours after steroid pulse. Error bars indicate S.D. [38].

3.3.6. Activated iNKT cells may damage the small-intestine allograft by release of IL-4/IL-5

Cytokine production by iNKT cells was observed in grafts and total cytokine concentration in sampled tissue was measured. Graphs show plots of the concentrations of IL-4, IL-5, IL-10, and IFN- γ (pg/mg in graft tissue) versus the mean number of IL-4 $^{+}$, IL-5 $^{+}$, IFN- γ^{+} , and IL-10 $^{+}$ -iNKT cells (per 10 high-power foci [HPF]). Linear regression lines and correlation coefficient (R^2) values are also shown (Figure 8).

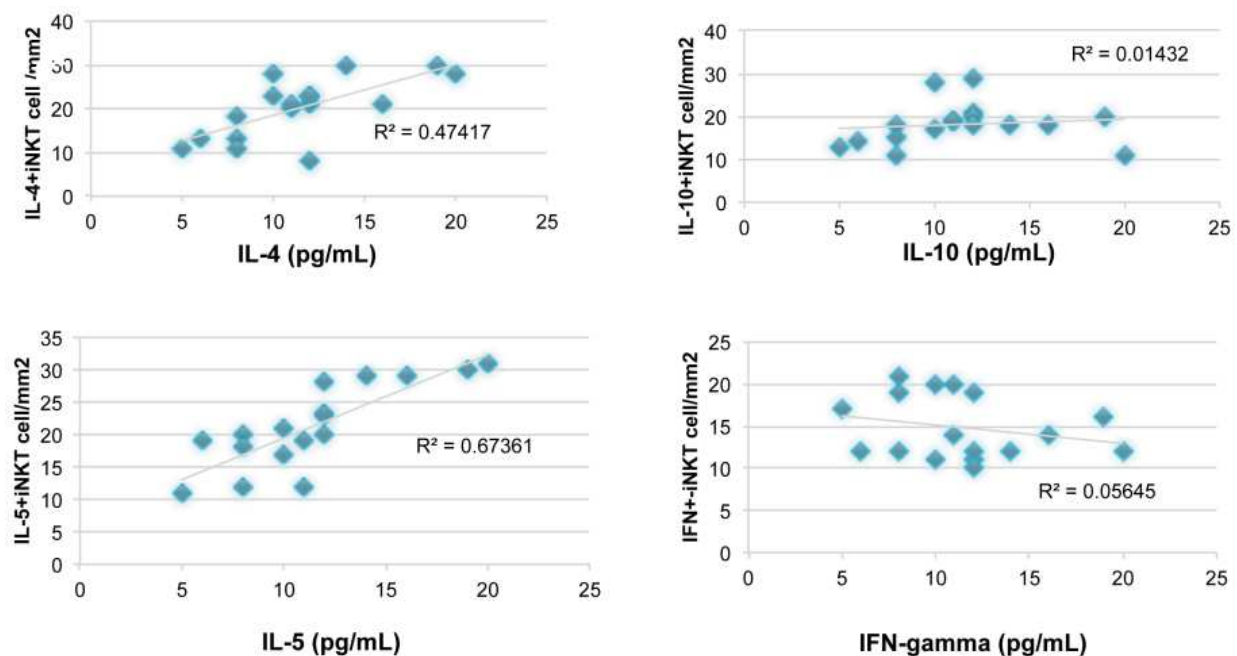


Figure 8. Regression analysis of cytokine concentration and counts of iNKT cells. IL-4 and IL-5 iNKT cell count correlate with total concentration of IL-4 and IL-5 in the graft.

It is likely that as yet unknown humoral factors recruit iNKT cells to the graft mucosa in order to suppress allograft rejection. iNKT cells have the ability to release IL-4 and antagonize Th1 and CTL responses [48]. However, because the released IL-4 and IL-5 may damage the allograft via eosinophilic enteritis, appropriate immunosuppression is necessary for recovery. We therefore investigated humoral rejection. iNKT cells may modulate such ACR via the release of cytokines.

3.4. NKT cells in intestinal inflammation caused by parasites

iNKT cells can also modulate mucosal immunity through the release of IL-4 and IL-5 in response to parasite infection. A previous study showed that NKT cells play a critical role in the lethal ileitis induced in C57BL/6 mice after infection with *Toxoplasma gondii* [49]. This intestinal inflammation is caused by overproduction of IFN- γ by iNKT cells in the LP. This detrimental activity of iNKT cells can be blocked by treatment with alpha-galactosylceramide, which induces a shift in cytokine production by iNKT cells toward a Th2 profile. iNKT cells participate in the clearance of parasites by shifting the cytokine profile toward a Th1 pattern. Another study using CD1d-deficient mice lacking iNKT cells showed that iNKT cells are critical for the elimination of *T. gondii* [50]. Thus, iNKT cells may form an immunological barrier against parasite infection.

4. iNKT cells in large-intestine immunity

4.1. NKT cells in the large intestine

Unlike the small intestine, a considerable number of NKT cells reside intra-epithelially in the colon of mice [9]. CD161⁺T cells are the major natural killer receptor-positive cell population in the intestine [10]. On the other hand, few reports have been published regarding human NKT cells, particularly iNKT cells, in the normal and neoplastic colon.

Our previous study was the first report the presence of TCRV α 24⁺ iNKT cells in the colon [29]. In that study, a small number of V α 24⁺NKT cells were observed in the normal colorectal mucosa (2.6 ± 3.7 cells/5 HPF), unlike the small intestine, which contained few resident NKT cells.

4.2. Intra-colorectal tumor iNKT cells

Furthermore, we investigated intra-tumor TCRV α 24-positive cells in 103 primary colorectal carcinoma samples [29]. The observation that intra-tumor V α 24⁺T cells invariably coexpress TCRV β 11 and other NK cell markers indicates that they are iNKT cells. The density of iNKT cells in colorectal carcinomas increased significantly. iNKT cells were actually found in the luminal space of tumor-infiltrating vessels. iNKT cells could migrate from the peripheral blood to the tumors. A higher fraction of iNKT cells express the activation marker CD69 in colorectal carcinomas than in normal mucosa, and these cells are probably involved in local cytotoxicity against tumor cells through secretion of IFN- γ and apoptosis-inducing molecules such as FasL, perforin, and granzyme B. In this way, iNKT cells may play a role in the primary defense against colorectal carcinoma by induction of tumor cell death. Alternatively, the increase in the number of NKT cells in tumors may represent a surrogate marker of antitumor activity.

Coca et al. reported that colorectal carcinomas with a higher degree of CD57⁺NK cell infiltration have better prognosis [51]. Because the CD57⁺cell population includes NKT cells, the present observation agrees in part with their conclusion. Another interesting feature of our study was the finding that iNKT cells play a role in the inhibition of lymph node metastasis of colorectal carcinomas [29]. Although iNKT cells are normally rare in lymph nodes, a large number of these cells appear in the metastasis-free swollen lymph nodes of colorectal carcinoma patients. The precise mechanism through which iNKT cells inhibit lymph node metastasis remains unclear. Activated iNKT cells may migrate to regional lymph nodes, where they may prevent metastasis by inducing apoptosis of the colorectal carcinoma cells. A recent study reported that chemokine CXCL16 suppresses liver metastasis of colorectal cancer via augmentation of tumor-infiltrating iNKT cells in a murine model [52].

Patients with a higher degree of intra-tumor NKT-cell infiltration show significantly higher rates of overall and disease-free survival. This is consistent with the fact that these patients exhibit much less lymph node metastasis. The degree of NKT cell infiltration in tumors was evaluated as low degree (<7 NKT cells/five x400 foci) or high degree (≥ 7 NKT cells/ five x400 foci). The number of iNKT cells increased markedly in colorectal carcinomas, and a majority of these cells showed a phenotype of activation expressing FasL or CD69. A higher degree of

iNKT cell infiltration correlated with lower lymph node metastasis ($P=0.042$). Patients with a high degree of iNKT cell infiltration showed higher overall ($P=0.018$) as well as disease-free ($P=0.0006$) survival rates. Intra-tumor iNKT cell infiltration was an independent prognostic factor for overall ($P=0.033$) and disease-free ($P=0.0064$) survival. A higher degree of iNKT infiltration in colorectal carcinomas is therefore an independent indicator of a favorable prognosis [29] (Figure 9).

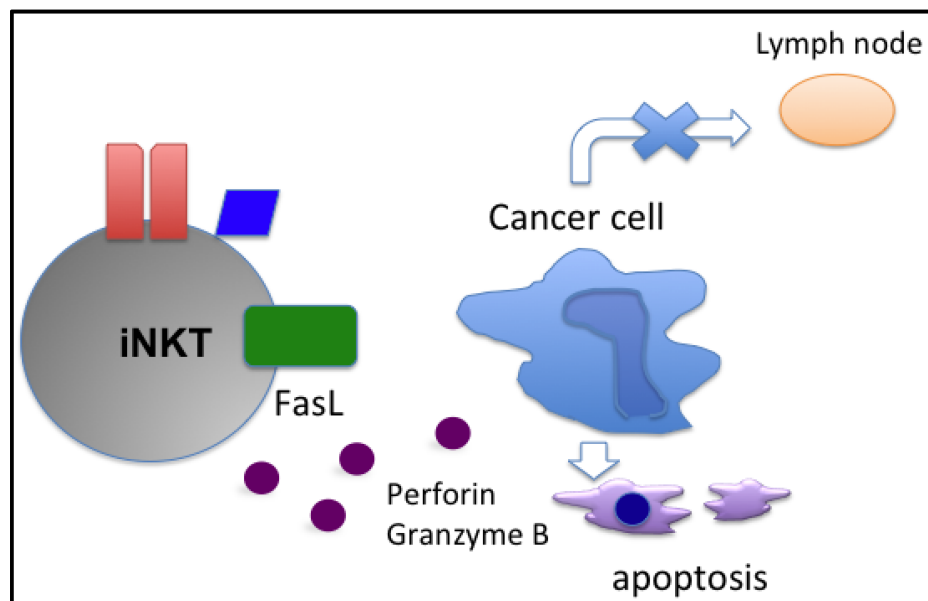


Figure 9. iNKT cells and apoptosis of tumor cells. FasL, perforin, and granzyme B are apoptosis-inducing molecules. iNKT cells may contribute to apoptosis of the cancer cell by release of these molecules and prevent the metastasis to the lymph node.

4.3. IBD and iNKT cells

The relevance of iNKT cells in IBD has also been examined. iNKT cells probably play an important role in the pathogenesis of ulcerative colitis [13, 22, 52]. Although our immunohistochemical data indicated that iNKT cells certainly contribute to inflammation, their influence is limited in the initial phase of IBD (data not shown). Several reports have indicated that iNKT cells secrete abundant amounts of IL-4 upon activation [53]. There has been a report that oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells [54].

Important data regarding the role of iNKT cells in IBD were recently reported [31]. iNKT cells accumulate in the colonic lamina propria in germ-free mice, resulting in increased morbidity in models of IBD as compared with specific pathogen-free mice. The chemokine ligand CXCL16 is associated with an increase in the number of mucosal iNKT cells and consequently an increase in susceptibility to tissue inflammation. Age-sensitive contact with commensal microbes is critical for establishing mucosal iNKT cell tolerance to later environmental exposures. Microbial exposure early in life elicits long-lasting effects on iNKT cells, and in the

absence of early exposure, exposure later in life to factors that stimulate these cells may induce an auto-inflammatory response.

In a related report, a profound loss of CD56 expression by all lymphocyte populations was noted in the coeliac gut. Adult coeliac disease expanded populations of CD3+TCR $\gamma\delta$ cells and decreased populations of NK, NKT, and iNKT cells [30].

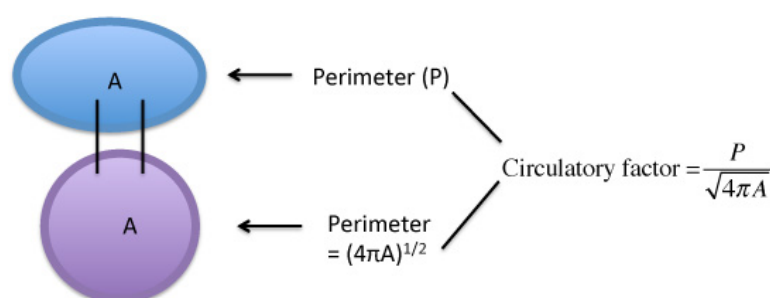
4.4. Microbial infection and iNKT cells

The relevance of iNKT cells against microbial infection has also been examined. CD1d-dependent antigen presentation and microbial killing by iNKT cells are critical for host defense [55]. The host defense against microbial infection depends upon cargo trafficking into lysosomes. The mechanism has been reported [56]. Arf-like GTPase Arl8b is one of the critical regulators of cargo delivery to lysosomes. The formation of CD1 antigen-presenting complexes in lysosomes and phagosome-lysosome fusion depends on Arl8b. Subsequently the delivered CD1 antigen-presenting complexes to the plasma membrane activate iNKT cells in microbial killing [56].

5. Methods

5.1. Quantitative morphometry

The quantitative morphometry method to study iNKT cells was applied (Figure 10). Multivariate morphometric analysis images were obtained by microscopy (PROVIS-AX80; Olympus, Tokyo, Japan) and saved as TIF files. Data were analyzed using CELAVIEW software (Olympus), with individual cell signals expressed in terms of relative fluorescent unit. Morphometric data were displayed according to stained area and circularity. Individual cells were represented by the pixel intensities of the nucleus and cytoplasm. Data sets for individual cells on a single slide were represented by two-dimensional scatter plots based on flow cytometry. Gated cells were further analyzed using antibodies for surface antigens. Plots were further gated according to fluorescence intensity, size, and circularity on the scattergram. Circularity was calculated based on the Heywood circularity factor using the following formula:



where the perimeter (P) of individual cells was divided by the circumference of a circle with the same area (A), with the real circle factor being equivalent to 1.0. As the boundary of a binary image is composed of discrete pixels, IMAQ Vision (National Instruments Corporation, Austin, TX, USA) was used to sub-sample the boundary [21, 39]. The scheme of quantitative morphometry is shown below.

Lymphocytes are oval or circular and the circulatory factor is nearly to 1.0. Therefore, the subjects of which circulatory factor is nearly to 1.0 is gated for subsequent analysis using immunostaining. iNKT cells were actually gated for subsequent analysis using antibodies for CD56 and TCRV α 24. In the first gating, the area was also analyzed [42] (Figure 10).

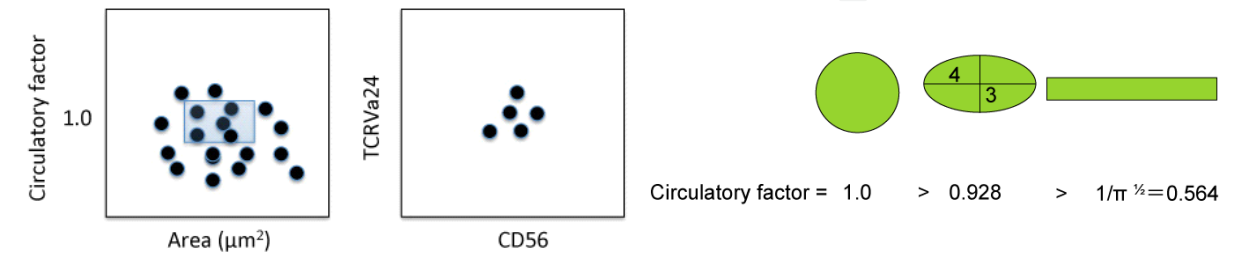


Figure 10. A scheme of two dimensional plots of the morphometric analysis for analysis of surface antigens CD56 and TCRV α 24 on the iNKT cells. The gating shown in the left graph shows an example of selection of the analyzed lymphocytes for analysis of the surface antigens shown in the right graph. The similarity to the circle is evaluated by the circulatory factor near 1.0. The right figures the factor values of various shapes.

5.2. Immunohistochemistry

The fluorescent staining method for iNKT cells was as reported using the CSA system (DAKO, Gostrup, Denmark). Frozen section samples and paraffin embedded specimens were used. For primary reagents, we used monoclonal antibody C15 (TCRV α 24; Immunotech SA, Marseilles, France). Antibodies against CD3, CD4 and CD8 were purchased from DAKO, and DAB staining was performed for signal visualization. Negative controls for primary antibodies were IgG1, IG2a and IgG2b (Cat No. X0931, X0943 and X0944; DAKO) [29, 38].

6. Conclusion

The mucosa is exposed to an extremely wide variety of antigens in food, and iNKT cells within the mucosa are responsible for mounting immune responses to these antigens. The cytokines released during these responses may damage the small intestine. In the case of small-intestine transplantation, the response of iNKT cells during ACR indicates that iNKT cells may be recruited to the transplant to repress rejection. iNKT cells probably contribute to the suppression of ACR by releasing IL-4, an antagonist of IFN- γ . On the other hand, iNKT cells normally reside within the large intestine, where they also play a pivotal role in immunity. iNKT cells can be activated to express apoptosis-inducing molecules and may induce cytolysis of tumor cells. Thus, iNKT cells have two roles; they can upregulate or downregulate the mucosal

immune response through the release of opposite-acting cytokines. iNKT cells thus play a pivotal role in modulating mucosal immunity in the intestine.

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Author details

Tatsuaki Tsuruyama and Wulamujiang Aini

Department of Diagnostic Pathology, Graduate School of Medicine, Kyoto University Hospital, Kyoto City, Japan

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