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Intestinal Intraepithelial TCRγδ$^+$ T Cells are Activated by Normal Commensal Bacteria

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TCRγδ$^+$ T cells play a critical role in protecting the intestinal mucosa against pathogenic infection. In the absence of infection, TCRγδ$^+$ T cell activation must be continuously regulated by T regulatory cells (Treg) to prevent the development of colitis. However, the activation of intestinal TCRγδ$^+$ T cells under normal conditions has not been clearly resolved. In order to determine TCRγδ$^+$ T cell activation *in vivo*, we designed an NF-κB based reporter system. Using the recombinant lentiviral method, we delivered the NF-κB reporter to isolated TCRγδ$^+$ T cells, which were then adoptively transferred into normal mice. Our data indicate that the NF-κB activation level in TCRγδ$^+$ T cells is higher in the intestinal intraepithelial layer than in the lamina propria region. In addition, the surface expression level of lymphocyte activation marker CD69 in TCRγδ$^+$ T cells is also higher in the intestinal intraepithelial layer and this activation was reduced by Sulfatrim treatment which removes of commensal bacteria. Collectively, our data indicate that the TCRγδ$^+$ T cell population attached to the intestinal lumen is constitutively activated even by normal commensal bacteria.

Keywords: TCRγδ$^+$ T cell, intestine, NF-κB, commensal bacteria

Introduction

Lymphocytes, which include B and T cells, play an essential role in the adaptive immune response. B cells are responsible for the humoral immune response, whilst T cells are involved in cell-mediated immune responses. T cells can be distinguished by the presence of a cell surface T-cell receptor (TCR), which is generated by the combinatorial rearrangement of different variable (V), diversity (D), and joining (J) segments (Hayday *et al.*, 1985; Garman *et al.*, 1986; Chien *et al.*, 1987). The majority of T cells in the periphery express a TCR composed of one α-chain and one β-chain. However, a subset of T cells possesses TCRs composed of γ- and δ-chains. In general, TCRαβ$^+$ T cells are essential for the adaptive immune response in the secondary lymphoid organ, while TCRγδ$^+$ T cells act as mediators between the innate and adaptive immune responses (Allison and Havran, 1991; Haas *et al.*, 1993; Holtmeier and Kabelitz, 2005). In terms of origin and development, however, TCRγδ$^+$ T cells are similar to TCRαβ$^+$ T cells. Both cell types are derived from common lymphoid progenitor cells and develop in the thymus, where progenitor cells are shared until the double negative 3 stage in thymopoiesis (Capone *et al.*, 1998; Livak *et al.*, 1999).

Antigen presenting cells (APCs) are required for presenting antigens to TCRαβ$^+$ T cells (Hayday *et al.*, 1985; Bonneville *et al.*, 2010). In contrast, it has been reported that certain populations of TCRγδ$^+$ T cells can recognize an antigen directly, without needing APC-mediated antigen presentation. In addition, some TCRγδ$^+$ T cells recognize the non-classical MHC class I b2-microglobulin (b2m)-associated molecules T10 and T22, while TCRαβ$^+$ T cells recognize typical class MHC I or class MHC II molecules (Yamashita *et al.*, 2005; Chien and Konigshofer, 2007).

Most TCRγδ$^+$ T cells develop in the thymus and then migrate to peripheral lymphoid organs (Pardoll *et al.*, 1988). As determined by the variable region in γ- and δ-chains, TCRγδ$^+$ T cells are divided into different subsets that are targeted to different organs (Bonneville *et al.*, 2010). At the site of activation, TCRγδ$^+$ T cells might act as important immune regulators in response to damage-mediated colitis, such as dextran sulfate sodium (DSS)-induced colitis and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (Chen *et al.*, 2002; Inagaki-Ohara *et al.*, 2004). However, a recent study suggested that TCRγδ$^+$ T cell activation exacerbates intestinal inflammation induced by dysregulated immune homeostasis (Park *et al.*, 2010). Yet it is well-established that TCRγδ$^+$ T cells do not elicit strong immune responses towards commensal bacteria that are normally present in the intestinal tract, despite playing a central role in the protective immune response triggered by pathogen infection in this region (Hayday, 2009). It has recently been suggested that regulatory T cell (Treg) is required to suppress the activation of intestinal TCRγδ$^+$ T cells in the absence of pathogen infection, and thereby to prevent colitis. However, the exact regulatory mechanism has not yet been resolved.

In this study, we sought to identify the precise site of TCRγδ$^+$ T cell activation *in vivo*. To this end, we designed a unique reporter system in which GFP expression is placed under the control of the NF-κB-regulated promoter. This system utilizes the fact that NF-κB activation is involved in TCRγδ$^+$ T cell activation, as well as in conventional T cell

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Abbreviations: TCR, t cell receptor; APC, antigen presenting cell; Treg, regulatory T cell; IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; LTR, long terminal repeat

activation (Park *et al.*, 2009; Turchinovich and Pennington, 2011). Expression of the T cell activation marker CD69 was also analyzed. We found that almost all TCRγδ$^+$ T cells displayed a highly activated phenotype at the intestinal intraepithelial layer, conveyed by a high level of NF-κB activation and CD69 expression at this site. Our data indicate that TCRγδ$^+$ T cells found in this layer are continuously activated.

Materials and Methods

Construction of the NF-κB-GFP lentiviral vector

Lentiviral plasmid DNA and pENTR^{TM} Directional TOPO Cloning Kits were purchased from Invitrogen (USA). The NF-κB reporter plasmid was constructed by inserting a DNA fragment containing 2 copies of the NF-κB binding site (ACA GAG GGG ACT TTC CGA GAG), and the c-*fos* basic promoter region (Howe *et al.*, 2002) upstream of the GFP open-reading frame. The DNA fragment was inserted into the pENTR vector and then a recombinant lentiviral vector was generated via recombination between pLenti6.2 (Invitrogen) and the newly constructed pENTR/NF-κB-GFP vector.

Infection of TCRγδ$^+$ T cells with recombinant lentivirus

Lentivirus infection was performed according to a modified version of the Invitrogen protocol. Briefly, 6.6×10^6 of 293FT cells were seeded on a 100-mm dish and cultured for 12 h in DMEM (Hyclone, USA) containing 10% fetal bovine serum (Hyclone), 2 mM l-glutamine (Gibco, USA), 0.1 mM non-essential amino acid solution (Gibco), and 1 mM sodium pyruvate solution (Gibco). The lentiviral vector (3 mg) and lentiviral packaging mix (9 mg) (Invitrogen) were combined, and the DNA was transfected into 293FT cells using Lipofectamine2000 (36 ml). After 12 h, the media were replaced and cells were then cultured for 48 h. The media were harvested and used for TCRγδ$^+$ T cell infection. Prior to infection, total intestinal lymphocytes were prepared (Weigmann *et al.*, 2007) and the cells were stained with anti-mouse TCRγδ. Among the cells, TCRγδ$^+$ T cells were sorted using FACS Aria (BD bioscience, USA). Uninfected cells were removed by blasticidin treatment.

Tissue sample preparation for imaging analysis

The lentivirus-infected TCRγδ$^+$ T cells were washed twice with PBS before re-suspending in 200 ml of fresh PBS. The cells were injected into C57BL/6 normal mice via intraperitoneal (IP) injection. After 2 days, frozen intestine tissue sections were prepared and 8–10-mm frozen sections were observed by fluorescence microscopy.

Isolation of intestinal intraepithelial and lamina propria lymphocytes

Intestinal intraepithelial lymphocytes (IELs) and intestinal lamina propria lymphocytes (LPLs) were prepared according to a modification of a method described previously (Weigmann *et al.*, 2007). Briefly, the intestine was washed with ice-cold PBS and incubated in IEL preparation buffer containing HBSS, 5% FBS, 1 mM DTT, and 5 mM EDTA at 37°C with shaking twice for 20 min. Cells were filtered using a 40-mM cell strainer and washed with 1× HBSS 3 times. The remaining intestinal tissues were incubated with digestion buffer containing PBS, 500 mg/ml of collagenase D, 500 mg/ml of DNase I, and 3 mg/ml of dispase II at 37°C by shaking twice for 20 min. Cells were filtered again through a 40-mM cell strainer, and prepared cells were washed with 1× HBSS 3 times. For the flow cytometry analysis, the lymphocytes were gated.

Results

Designing and testing the *in vivo* NF-κB-GFP recombinant lentiviral reporter system

During conventional T-cell stimulation, NF-κB activation is required for cytokine gene expression and activated T cell survival (Hayden and Ghosh, 2011). In TCRγδ$^+$ T cells,

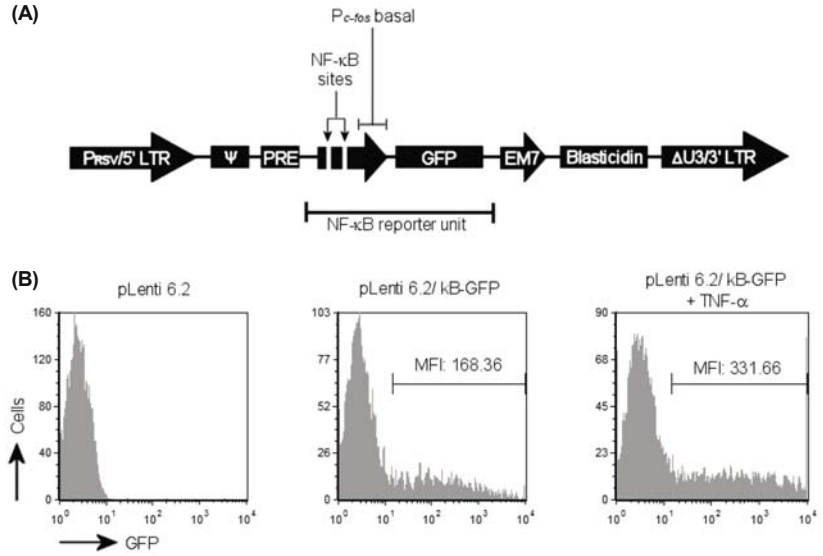

Fig. 1. Construction of a recombinant lentiviral vector for the detection of NF-κB activation *in vivo*. (A) The pLenti6.2/NF-κB-GFP reporter lentiviral vector was constructed by insertion of the NF-κB promoter and GFP open-reading frame into the pLenti6.2 lentiviral vector (B) Hep3B cells were transfected with pLenti6.2/NF-κB-GFP reporter plasmids, and the cells were treated with TNF-α. The cells were analyzed by flow cytometry. MFI: mean fluorescence intensity.

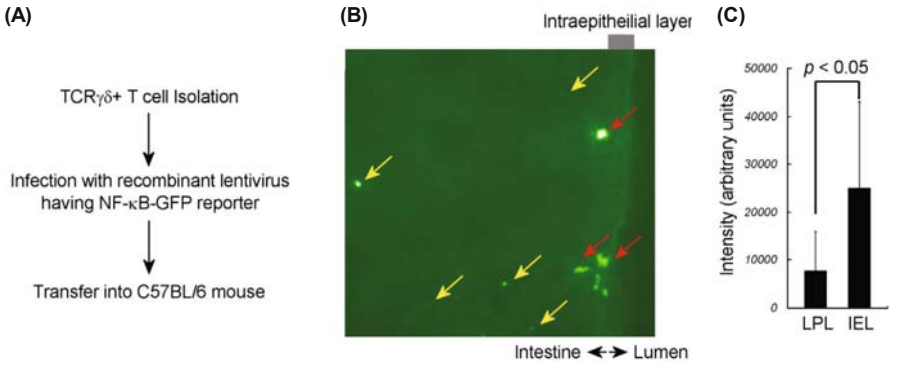

Fig. 2. NF-κB activation of TCRγδ+ T cells in intestinal tissues (A) Summary of the experimental procedure (B) TCRγδ+ T cells were infected with recombinant lentiviruses containing the NF-κB-GFP cassette. Cells were treated with blasticidin to remove uninfected TCRγδ+ T cells. Infected TCRγδ+ T cells were transferred into a normal C57BL/6 mouse and intestinal tissue sections were visualized by fluorescence microscopy. The yellow arrow indicates TCRγδ+ T cells in the lamina propria region, and the red arrow shows TCRγδ+ T cells localized to the intraepithelial layer. (C) Intensities of the GFP signals were analyzed using Image Gauge software (Fuji). Statistical analysis was performed using the two-tailed Student's *t* test, with unequal variance.

NF-κB is also activated during cell activation (Turchinovich and Pennington, 2011). Therefore, NF-κB activation can reflect the activation of TCRγδ+ T cells. In this study, to check the TCRγδ+ T cell activation region in *in vivo*, we designed

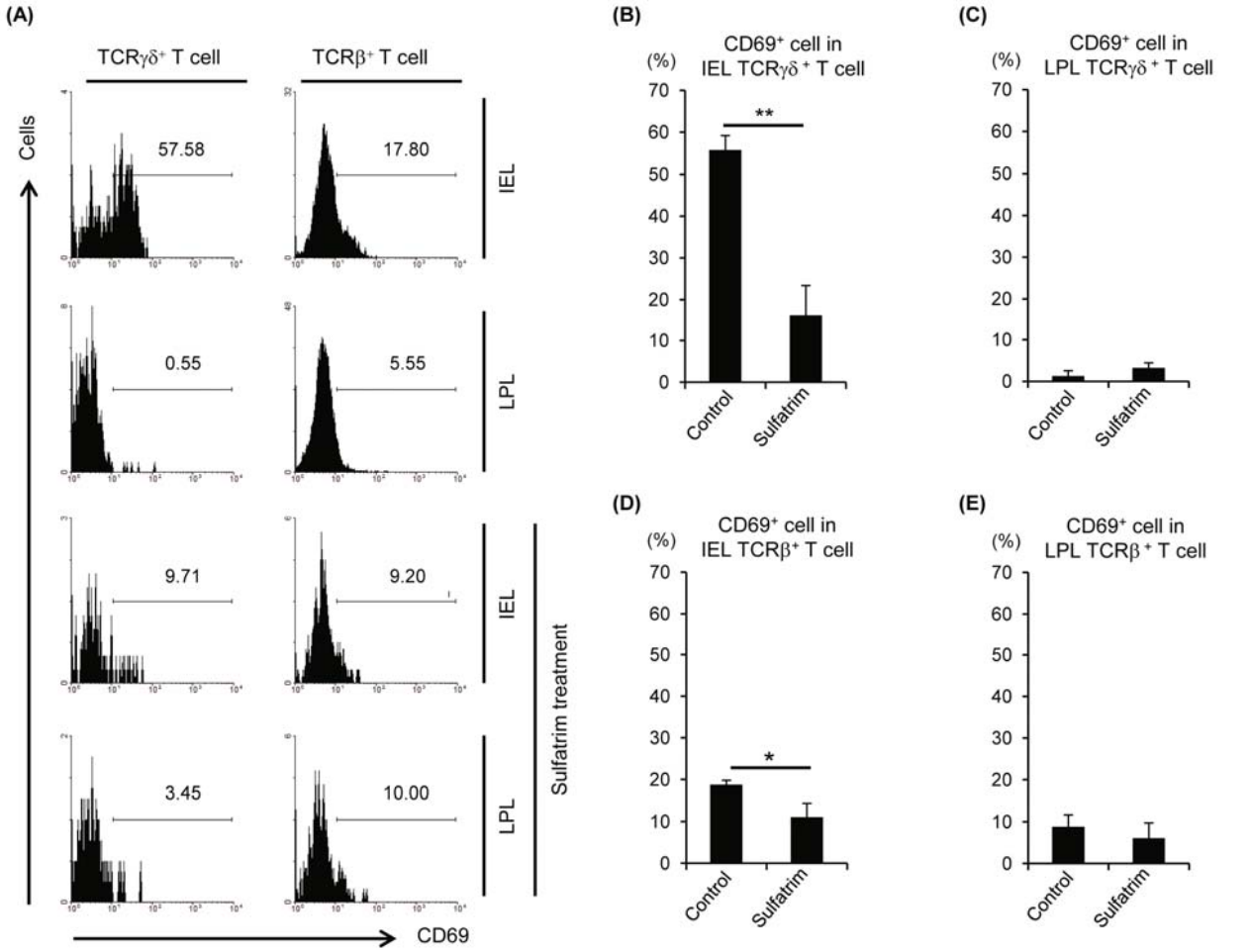

Fig. 3. CD69 expression on T cells from the intestinal intraepithelial layer and lamina propria region. IELs and LPLs were isolated from the normal C57BL/6 mouse as described in 'Materials and Methods'. (A) The isolated lymphocytes were stained with anti-mouse CD69, anti-mouse TCRb, and anti-mouse TCRγδ antibodies. The stained cells were analyzed by flow cytometry. In this experiment, for removing of intestinal commensal bacteria, mice were treated by Sulfatrim for 2 weeks. (B, C, D, and E) CD69+ population of TCRγδ+ T cells (B and C) or TCRb+ T cells (D and E) in IEL and LPL were analyzed (n =3 mice). Statistical analysis was performed using the two-tailed Student's *t* test, with unequal variance. **p*<0.05, ***p*<0.01.

an NF-κB activation reporter system in which GFP expression is under the control of an NF-κB-regulated promoter. To overcome the difficulties associated with gene delivery into primary TCRγδ$^+$ T cells, we utilized the recombinant lentiviral system. Thus, we inserted the NF-κB-GFP reporter cassette into a lentiviral vector (Fig. 1A).

To verify that the newly constructed NF-κB-GFP recombinant lentiviral vector can respond to NF-κB stimulation, we transfected the vector into Hep3B cells and treated the transfected cells with TNF-α, a well-known stimulus for NF-κB activation. Subsequent flow cytometry analysis indicated positive GFP signals in transfected cells in the absence of TNF-α stimulation. In addition, the intensity of these signals was observed to increase in response to TNF-α treatment (Fig. 1B). It has previously been shown that the 5′ long terminal repeats (LTR) can act as RNA polymerase II promoter regions. Thus, basal GFP expression may be a consequence of basal promoter activity associated with the 5′LTR. In addition to TNF-α stimulation, we also found that the GFP signal intensity was increased by treatment with lipopolysaccharide, which is another stimulus for NF-κB activation (data not shown).

The NF-κB activation level in intestinal intraepithelial TCR γδ$^+$ T cells is higher than that in lamina propria TCRγδ$^+$ T cells

To determine the region of TCRγδ$^+$ T cell activation in the intestine, we infected TCRγδ$^+$ T cells with the NF-κB-GFP recombinant lentivirus. Transduced cells were treated with blasticidin for 1.5 days to remove uninfected TCRγδ$^+$ T cells, and selected cells were then transferred into a normal C57BL/6 mouse. After 2 days, the intestinal tissue section was prepared and visualized by fluorescence microscopy. A GFP signal was observed in the intestinal sample from mouse injected with NF-κB-GFP recombinant lentivirus-infected TCRγδ$^+$ T cell but not in the tissue sample prepared from a control mouse (data not shown). Interestingly, the GFP signal intensity was higher in the intraepithelial layer than in the lamina propria region (Fig. 2B). These data indicate that NF-κB activity is higher in the intestinal intraepithelial TCRγδ$^+$ T cell population than in the intestinal lamina propria TCRγδ$^+$ T cell population. The data suggest that NF-κB in TCRγδ$^+$ T cells are constitutively activated in the intestinal intraepithelial region.

Intestinal intraepithelial TCRγδ$^+$ T cells are activated by intestinal commensal bacteria

The surface expression level of CD69 is increased in activated T cells. Therefore, the expression of this marker can be tracked as an alternative measure of T cell activation. To determine whether the expression patterns of CD69 in intestinal TCRγδ$^+$ T cells similar to the NF-κB activation results described above, we assessed the CD69 surface expression in TCRγδ$^+$ T cells obtained from the intestinal intraepithelial region and lamina propria region (Figs. 3A, 3B, and 3C). CD69 surface expression levels were also determined in TCRαβ$^+$ T cells present in each region (Figs. 3A, 3D, and 3E). Our analysis revealed high expression of CD69 in intestinal intraepithelial TCRγδ$^+$ T cells but not in intestinal lamina propria TCRγδ$^+$ T cells. Furthermore, TCRαβ$^+$ T cells did not highly express CD69, either in the intestinal intraepithelial region or in the lamina propria region (Figs. 3A and 3D). Taken together, these data indicate that TCRγδ$^+$ T cells, but not TCRαβ$^+$ T cells, are highly activated in the intraepithelial layer of intestinal tissues. In addition, removing of intestinal commensal bacteria by sulfatrim treatment dramatically reduced CD69 expression on intestinal intraepithelial TCRγδ$^+$ T cells (Figs. 3A and 3B). Thus, the data indicated that TCRγδ$^+$ T cells were activated by intestinal commensal bacteria even under normal conditions.

Discussion

TCRγδ$^+$ T cells constitute a minor T cell population in the periphery, but these cells are enriched in the intestine, lungs, and skin. TCRγδ$^+$ T cells are important for epithelial cell regeneration and for the elicitation of an early immune response to infectious pathogens (Hayday and Tigelaar, 2003; Bonneville *et al.*, 2010). In spite of this protective role in the immune system, many recent studies have provided evidence that TCRγδ$^+$ T cell activation underlies inflammatory diseases such as multiple sclerosis, asthma, and colitis (Ito *et al.*, 2009; Sutton *et al.*, 2009; Park *et al.*, 2010). In multiple sclerosis and asthma, TCRγδ$^+$ T cells secrete inflammatory cytokines, particularly IL-17, which mediates inflammation. However, cytokine secretion is an important process involved in pathogen clearance during an infection. Thus, regulation of TCRγδ$^+$ T cell activation is crucial for preventing the occurrence of an unnecessary immune response without compromising intrinsic protective mechanisms.

The role of TCRγδ$^+$ T cells in the intestine is complex and has been debated in the literature. Some papers report an immune-regulatory function of TCRγδ$^+$ T cells while others describe an inflammation-initiating function. During damage-mediated inflammation in the intestine, TCRγδ$^+$ T cells have been shown to perform an important immune-regulatory function. However, TCRγδ$^+$ T cells have also been shown to induce spontaneous colitis in mice in the absence of an immune regulator such as Treg. In conditions of intestinal damage, TCRγδ$^+$ T cells might be involved in inflammatory responses responsible for clearing bacteria that have been disseminated following disruption of the intestinal intraepithelial cell barrier. This might explain why damage-mediated intestinal inflammation is more severe in the absence of TCRγδ$^+$ T cells. However, it remains unclear why TCRγδ$^+$ T cells might induce spontaneous intestinal inflammation in cells lacking Treg. The data presented here might provide an insight into the underlying mechanism.

A previous study showed that activation marker expression levels on TCRγδ$^+$ T cells are higher in intestinal IELs than in intestinal LPLs (Park *et al.*, 2010). Nonetheless, the preparation of IELs and LPLs is a time-consuming procedure with many steps, during which the original *in vivo* state of TCRγδ$^+$ T cells could be altered. Thus, we designed a unique reporter system that could be used to measure the activation level of TCRγδ$^+$ T cells *in vivo*. Our data show that TCRγδ$^+$ T cell activation is enriched in the intestinal intraepithelial region compared with the lamina propria region.

This finding indicates that TCRγδ+ T cells are activated in response to commensal bacteria in the intestinal lumen. Hence, TCRγδ+ T cells are activated constitutively within this intestinal layer, and this could provide a mechanism for the induction of intestinal inflammation by TCRγδ+ T cells in the absence of regulation by Treg.

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References

Allison, J.P. and Havran, W.L. 1991. The immunobiology of T cells with invariant gamma delta antigen receptors. *Ann. Rev. Immunol.* **9**, 679–705.

Bonneville, M., O'Brien, R.L., and Born, W.K. 2010. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat. Rev. Immunol.* **10**, 467–478.

Capone, M., Hockett, R.D., Jr., and Zlotnik, A. 1998. Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)CD25(+) Pro-T thymocytes. *Proc. Natl. Acad. Sci. USA* **95**, 12522–12527.

Chen, Y., Chou, K., Fuchs, E., Havran, W.L., and Boismenu, R. 2002. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc. Natl. Acad. Sci. USA* **99**, 14338–14343.

Chien, Y.H., Iwashima, M., Wettstein, D.A., Kaplan, K.B., Elliott, J.F., Born, W., and Davis, M.M. 1987. T-cell receptor delta gene rearrangements in early thymocytes. *Nature* **330**, 722–727.

Chien, Y.H. and Konigshofer, Y. 2007. Antigen recognition by gammadelta T cells. *Immunol. Rev.* **215**, 46–58.

Garman, R.D., Doherty, P.J., and Raulet, D.H. 1986. Diversity, rearrangement, and expression of murine T cell gamma genes. *Cell.* **45**, 733–742.

Haas, W., Pereira, P., and Tonegawa, S. 1993. Gamma/delta cells. *Ann. Rev. Immunol.* **11**, 637–685.

Hayday, A.C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* **31**, 184–196.

Hayday, A.C., Saito, H., Gillies, S.D., Kranz, D.M., Tanigawa, G., Eisen, H.N., and Tonegawa, S. 1985. Structure, organization, and somatic rearrangement of T cell gamma genes. *Cell.* **40**, 259–269.

Hayday, A. and Tigelaar, R. 2003. Immunoregulation in the tissues by gammadelta T cells. *Nat. Rev. Immunol.* **3**, 233–242.

Hayden, M.S. and Ghosh, S. 2011. NF-kappaB in immunobiology. *Cell Res.* **21**, 223–244.

Holtmeier, W. and Kabelitz, D. 2005. Gammadelta T cells link innate and adaptive immune responses. *Chem. Immunol. Allergy* **86**, 151–183.

Howe, C.J., LaHair, M.M., Maxwell, J.A., Lee, J.T., Robinson, P.J., Rodriguez-Mora, O., McCubrey, J.A., and Franklin, R.A. 2002. Participation of the calcium/calmodulin-dependent kinases in hydrogen peroxide-induced Ikappa B phosphorylation in human T lymphocytes. *J. Biol. Chem.* **277**, 30469–30476.

Inagaki-Ohara, K., Chinen, T., Matsuzaki, G., Sasaki, A., Sakamoto, Y., Hiromatsu, K., Nakamura-Uchiyama, F., Nawa, Y., and Yoshimura, A. 2004. Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation. *J. Immunol.* **173**, 1390–1398.

Ito, Y., Usui, T., Kobayashi, S., Iguchi-Hashimoto, M., Ito, H., Yoshitomi, H., Nakamura, T., Shimizu, M., Kawabata, D., Yukawa N., and *et al.* 2009. Gamma/delta T cells are the predominant source of interleukin-17 in affected joints in collagen-induced arthritis, but not in rheumatoid arthritis. *Arthritis Rheum.* **60**, 2294–2303.

Livak, F., Tourigny, M., Schatz, D.G., and Petrie, H.T. 1999. Characterization of TCR gene rearrangements during adult murine T cell development. *J. Immunol.* **162**, 2575–2580.

Pardoll, D.M., Fowlkes, B.J., Lew, A.M., Maloy, W.L., Weston, M.A., Bluestone, J.A., Schwartz, R.H., Coligan, J.E., and Kruisbeek, A.M. 1988. Thymus-dependent and thymus-independent developmental pathways for peripheral T cell receptor-gamma delta-bearing lymphocytes. *J. Immunol.* **140**, 4091–4096.

Park, S.G., Mathur, R., Long, M., Hosh, N., Hao, L., Hayden, M.S., and Ghosh, S. 2010. T regulatory cells maintain intestinal homeostasis by suppressing gammadelta T cells. *Immunity* **33**, 791–803.

Park, S.G., Schulze-Luehrman, J., Hayden, M.S., Hashimoto, N., Ogawa, W., Kasuga, M., and Ghosh, S. 2009. The kinase PDK1 integrates T cell antigen receptor and CD28 coreceptor signaling to induce NF-kappaB and activate T cells. *Nat. Immunol.* **10**, 158–166.

Sutton, C.E., Lalor, S.J., Sweeney, C.M., Brereton, C.F., Lavelle, E.C., and Mills, K.H. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* **31**, 331–341.

Turchinovich, G. and Pennington, D.J. 2011. T cell receptor signalling in gammadelta cell development: strength isn't everything. *Trends Immunol.* **32**, 567–573.

Weigmann, B., Tubbe, I., Seidel, D., Nicolaev, A., Becker, C., and Neurath, M.F. 2007. Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nature Protocols* **2**, 2307–2311.

Yamashita, S., Tanaka, Y., Tsutsumi, S., Aburatani, H., Minato, N., and Ihara, S. 2005. Analysis of mechanism for human gammadelta T cell recognition of nonpeptide antigens. *Biochem. Biophys. Res. Commun.* **334**, 349–360.