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-xB based reporter system. Using the recombinant lentiviral method, we delivered the NF-xB reporter to isolated TCRγδ+ T cells, which were then adoptively transferred into normal mice. Our data indicate that the NF-xB 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 than in the lamina propria region. The majority of TCRγδ+ T cells in the periphery express 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 1 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-xB-regulated promoter. This system utilizes the fact that NF-xB activation is involved in TCRγδ+ T cell activation, as well as in conventional T cell

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,

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

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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™ 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, 6.6×10⁶ 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.

**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, 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.

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