Increase of CD4⁺CD25⁺ T cells in Smad3⁻/⁻ mice

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Abstract

AIM: To investigate the changes of lymphocyte subpopulations, especially CD4⁺CD25⁺ T regulatory cells in Smad3⁻/⁻ mice.

METHODS: Hematological changes and changes of lymphocyte subpopulations were detected in Smad3⁻/⁻ mice using cell counter and flow cytometry, respectively, and compared to their littermate controls.

RESULTS: The numbers of neutrophils and lymphocytes in peripheral blood were significantly increased in Smad3⁻/⁻ mice compared to littermate controls. CD19⁺ expressing cells in blood and spleen, and CD8⁺ T cells in thymus were all markedly decreased in Smad3⁻/⁻ mice. More important, Smad3⁻/⁻ mice had an increased population of CD4⁺CD25⁺ T cells in peripheral lymphoid tissues, including thymus, spleen, and lymph nodes.

CONCLUSION: These observations suggest that the changes of lymphocyte subpopulations might play a role in susceptibility to inflammation of Smad3⁻/⁻ mice.

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Key words: CD4⁺CD25⁺ T cells; Lymphocyte subpopulation; SMAD3; TGF-β signaling

INTRODUCTION

TGF-β plays an important role in maintaining immune homeostasis. It signals through a set of transmembrane receptor serine/threonine kinases unique to the large superfamily of TGF-β related proteins [1]. As a downstream cytoplasm signaling element of TGF-β receptors, Smad3 mediates a positive signal pathway from the receptor serine/threonine kinases to the nuclei [2]. Previous reports revealed Smad3 plays an important role in mediating TGF-β signal in T lymphocytes and neutrophils, and demonstrated that Smad3 deficiency results in immune dysregulation and susceptibility to opportunistic infection [3].

The immune system discriminates between self and non-self, establishing and maintaining unresponsiveness to self. There is clear evidence that clonal deletion of self-reactive T and B cells is a major mechanism of self-tolerance [4]. However, the fact that potentially hazardous self-reactive lymphocytes are present in the periphery of normal adult individuals [5] reveals that the mechanisms that can prevent pathological autoimmunity exist. In recent years, a burst of papers are focused on a population of CD4⁺ T cells that constitutively express the IL-2Rα (CD25) T cells and reveal them as key “actors” to self-tolerance [6]. A direct experiment to assess the regulatory role of CD4⁺CD25⁺ T cells in self-tolerance reported that the adoptive transfer of CD4⁺CD25⁺-depleted T cells could induce several organ-specific autoimmune diseases in immunodeficient animals [7]. CD4⁺CD25⁺ T cells also regulate antibody responses against self- and non-self-antigens by direct inhibitory effects on B cells or via inhibition of Th cell differentiation [8, 9]. In addition to self-tolerance and autoimmunity, there is evidence that CD4⁺CD25⁺ T cells are actively engaged in negative control of a broad spectrum of immune responses induced by microbial infection [10-13]. They can also mediate transplantation tolerance [14] and maternal tolerance to the foetus [15].

Although great progress in CD4⁺CD25⁺ T cells study has been made in recent years, many issues remain to be solved. For example, the involvement of TGF-β in CD4⁺CD25⁺ T cell immunoregulatory function is still controversial [16-18]. In the present study, we examined the changes of lymphocyte subpopulations in peripheral lymphoid tissues of Smad3⁻/⁻ mice as well as their controls. Our results showed that Smad3⁻/⁻ mice were associated with an increased population of CD4⁺CD25⁺ T cells, suggesting that CD4⁺CD25⁺ T cells might play a role in susceptibility to inflammation of Smad3⁻/⁻ mice.

MATERIALS AND METHODS

Mice

Smad3⁻/⁻ mice were generated by targeted gene disruption...
in murine embryonic stem cells by homologous recombination \(^9\). Both Smad3\(^{-/-}\) mice and their littermate controls (wild-type, Smad3\(^{+/+}\)) were provided by Xiao Yang (Institute of Biotechnology, Beijing, China). The mice used in these experiments were 6-8 wk of age.

**Antibodies and reagents**

PE-anti-CD4, FITC-anti-CD8, FITC-anti-CD3, and PE-anti-CD19 were purchased from Southern Biotechnology Associates (Birmingham, USA). FITC-anti-CD25 was purchased from Biolegend (San Diego, CA).

**Analysis of leukocytes in peripheral blood**

Before mice were sacrificed, approximately 20 μL blood samples were collected through tail vein, diluted, and then analyzed on Sysmex F-820 semi-automatic analyzer (Japan).

**Flow cytometry of lymphocytes**

Peripheral blood, thymus, spleen and lymph nodes were harvested from mice. Single-cell suspensions were subjected to hypotonic lysis of red blood cells (Becton Dickinson), washed in phosphate-buffered saline, stained with fluorescein-conjugated antibodies according to standard protocols, and then analyzed on an FACSScan (Beckman Dickinson). For isolation of peripheral blood mononuclear cells (PBMC), 2 mL of heparinised peripheral blood diluted 1:1 with PBS was layered onto an equal volume of Ficoll-Hypaque density gradient solution and centrifuged at 300 r/min at room temperature. The mononuclear cells were collected, washed twice with PBS.

**Statistical analysis**

Difference was defined as being statistically significant when \(P < 0.05\) was obtained using Student’s \(t\) test.

**RESULTS**

**Increased numbers of neutrophils and lymphocytes in Smad3\(^{-/-}\) mice**

We first compared the total numbers of white blood cells and differential distributions of leukocytes in peripheral blood samples from Smad3\(^{-/-}\) mice and littermate control mice. A marked increase in absolute white blood cell counts was observed in Smad3\(^{-/-}\) mice (\(P < 0.01\)). Accordingly, the numbers of neutrophils and lymphocytes were also elevated in Smad3\(^{-/-}\) mice compared to their controls (Figure 1). These results are consistent with a previous report that Smad3\(^{-/-}\) mice exhibited invasive mucosal infection involving multiple immune organs \(^9\).

**Changes of lymphocyte subpopulations**

Susceptibility of Smad3\(^{-/-}\) mice to infection and tissue inflammation \(^9\) made us wonder whether quantitative changes of lymphocytes were present in these mice. The results showed that numbers of CD19\(^+\)-expressing cells (most B cells) in the peripheral blood and spleen were significantly decreased in Smad3\(^{-/-}\) mice compared to their controls (Figures 2A and 2C). In addition, the number of CD8\(^+\) T cells was also reduced in thymus in Smad3\(^{-/-}\) mice (Figure 2B). Analysis of lymph nodes did not reveal any significant difference between the mutant mice and littermate controls (Figure 2D).

![Figure 1](image_url)  
**Figure 1** Total numbers and differential distributions of blood leukocytes in Smad3\(^{-/-}\) and Smad3\(^{+/+}\) mice. Peripheral blood samples were collected from tail veins of mice, and then analyzed on Sysmex F-820. Shown here are the means and standard deviations of total numbers and distributions of blood leukocytes from 4 wild-type and 4 mutant mice (\(\*P < 0.05\), \(\*\*P < 0.01\)).

![Figure 2](image_url)  
**Figure 2** Percentage of lymphocyte subpopulations in peripheral blood (A), thymus (B), spleen (C) and lymph nodes (D) of wild-type and mutant mice. The cells were stained with PE-anti-CD4 and FITC-anti-CD8, or with FITC-anti-CD3 and PE-anti-CD19, and then subjected to cytometric analyses. Shown here are the means and standard deviations of percentage of lymphocyte subpopulations from 4 wild-type and 4 mutant mice (\(\*P < 0.05\), \(\*\*P < 0.01\)).
Increased CD4⁺CD25⁺ T cells in Smad3⁻/⁻ mice

CD4⁺CD25⁺ T cells play an important role in maintaining the equilibrium between immunity and tolerance. Many papers have reported that this population of cells is able to suppress proliferation and effector function of CD4⁺ and CD8⁺ T cells. To explore whether the decreased lymphocytes in Smad3⁻/⁻ mice were related to the CD4⁺CD25⁺ T cells, we examined this population of cells in peripheral lymphoid tissues of Smad3⁻/⁻ mice and littermate controls. Smad3⁻/⁻ mice exhibited a greater percentage of CD4⁺CD25⁺ T cells in thymus, spleen and lymph nodes, compared to controls. In peripheral blood, however, no difference was observed between mutant mice and wild type in regards to CD4⁺CD25⁺ T cell proportion (Figure 3).

DISCUSSION

TGF-β is an essential endogenous regulator of T-cell function. It has been recently reported that TGF-β reporter mice have normal numbers of CD4⁺CD25⁺ T cells after birth, indicating that CD4⁺CD25⁺ T cells are able to develop in complete absence of endogenous TGF-β expression. This made us think whether quantitative or functional changes of CD4⁺CD25⁺ T cells occurred in Smad3⁻/⁻ mice. Our main finding in this study is that Smad3⁻/⁻ mice had increased CD4⁺CD25⁺ T cells compared to their littermate controls (Figure 3). Our results showed that neutrophil and lymphocyte numbers increased (Figure 1) and that lymphocyte population decreased in the peripheral lymphoid tissues of Smad3⁻/⁻ mice (Figure 2), which are consistent with the previous reports.

During infection, the balance between self-reactive effector T cells and regulatory T cells could determine the time of onset, the intensity and duration of autoimmune response. Recent studies have focused on a population of CD4⁺ T cells that constitutively express CD25. CD4⁺CD25⁺ T cells comprise 5%-10% of the peripheral CD4⁺ T cell pool of normal mice and humans and exhibit immunosuppressive abilities both in vitro and in vivo. Studies of human diseases indicate that the functional CD4⁺CD25⁺ T cells are enriched in inflamed joints of patients with rheumatoid arthritis or with the juvenile idiopathic arthritis. In our study, we showed that an increased population of CD4⁺CD25⁺ T cells was present in Smad3⁻/⁻ mice, which could partially account for the susceptibility to inflammation of these mutant mice. However, our results and those of a previous study did not reveal any significant difference of CD4⁺ T cells of spleen and lymph nodes between the asymptomatic mice and littermate controls. A possible explanation for the increase of CD4⁺CD25⁺ T cells is that they are derived from the CD4⁺CD25⁺ cells under the condition of Smad3 gene mutation. It is well accepted that CD4⁺CD25⁺ T cells can be generated by the activation of mature, peripheral CD4⁺CD25⁺ T cells under different stimulatory conditions. Further studies should concentrate on defining the functional characteristics of the CD4⁺CD25⁺ T cells in Smad3⁻/⁻ mice to gain a better insight into the mechanisms of susceptibility to inflammation.

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