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Dysregulation of CD1d-Restricted Type II Natural Killer T Cells Leads to Spontaneous Development of Colitis in Mice

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Abstract

Background & Aims—CD1d-restricted natural killer (NK) T cells are a subset of immunoregulatory T cells that comprise type I (express the semi-invariant T-cell receptor [TCR] and can be detected using the α -GalCer/CD1d tetramer) and type II (express diverse TCRs and cannot be directly identified). Studies in mouse models of inflammatory bowel disease revealed a complex role for type I NKT cells in the development of colitis. Type II NKT cells have been associated with intestinal inflammation in patients with ulcerative colitis.

Method—To investigate whether dysregulation of type II NKT cells, caused by increased expression of CD1d, can contribute to colitis, we generated transgenic mice that express high levels of CD1d and a TCR from an autoreactive, type II NKT cell (CD1dTg/24 α β Tg mice).

Results—CD1dTg/24 α β Tg mice had reduced numbers of 24 α β T cells, compared with 24 α β Tg mice, indicating that negative selection increases among type II NKT cells engaged by abundant self-antigen. The residual 24 α β T cells in CD1dTg/24 α β Tg mice had an altered surface phenotype and acquired a cytokine profile distinct from that of equivalent cells in 24 α β Tg mice. Interestingly, CD1dTg/24 α β Tg mice spontaneously developed colitis; adoptive transfer experiments confirmed that type II NKT cells that develop in the context of increased CD1d expression are pathogenic.

Conclusions—Aberrant type II NKT cell responses directly contribute to intestinal inflammation in mice, indicating the importance of CD1d expression levels in the development and regulation of type II NKT cells.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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Keywords

IBD; Crohn's disease; immune regulation; T-cell development

INTRODUCTION

CD1d molecules are MHC class I-like cell surface glycoproteins that are specialized to present self and microbial lipid antigens to NKT cells. NKT cells exhibit an activated phenotype such that upon TCR engagement, NKT cells rapidly produce an array of cytokines, which can influence both innate and adaptive immune responses.¹ The CD1d-restricted NKT cell population can be divided into two subsets based on their reactivity to α -galactosylceramide (α -GalCer).^{2,3} Type I NKT cells, or invariant NKT (iNKT) cells, express a highly restricted TCR repertoire (an invariant V α 14-J α 18 paired predominantly with V β 8.2 in mice, and V α 24-J α 18 paired with V β 11 in humans)⁴, are reactive to α -GalCer, and can bind to CD1d/ α -GalCer tetramer.⁵⁻⁷ Type II NKT cells, also referred to as variant NKT (vNKT) cells, express diverse TCR α and β chains and do not respond to α -GalCer.^{8,9} Recent studies have shown that a fraction of type II NKT cells respond to sulfatide, a self-glycolipid that is abundant in myelin sheath.¹⁰⁻¹² However, CD1d/sulfatide tetramer has not been widely used to characterize type II NKT cells due to its high background staining and low stability. Although type I NKT cells are the dominant NKT cell population in mice, in humans type II NKT cells appear to be more prevalent than type I NKT cells.¹³ The differences between type I and type II NKT cells in TCR usage, and consequently in their distinct ligand recognition, suggest that these NKT cell subsets may play distinct immunological roles. Indeed, the contrasting roles of type I and type II NKT cells in tumor immunity have been described.¹⁴

Because NKT cell activation can prevent or promote immunopathology in various disease settings, both NKT cell selection and development appear to be tightly regulated. We and others have demonstrated that type I NKT cells undergo negative selection when engaged by high-avidity antigen or abundant self-antigen.^{15,16} In addition, we found that increased CD1d expression on dendritic cells, but not on TEC, leads to the enhanced negative selection of type I NKT cells.¹⁵ Moreover, overexpression of CD1d on thymocytes and peripheral T cells using the *Lck* promoter resulted in a reduced percentage and altered function of type I NKT cells compared to those found in wild type mice, suggesting that changes in the CD1d expression pattern can modulate the development and function of type I NKT cells.¹⁷ In contrast to type I NKT cell development, little is known regarding type II NKT cell development due to the lack of surface markers that specifically define this T cell subset.

Recent studies have shown that CD1d expression levels are altered under inflammatory conditions as well as during infection.^{18,19} Due to the autoreactive nature of type I and type II NKT cells, it is likely that changes in CD1d expression levels during infection/inflammation could influence the extent of NKT cell responses, irrespective of whether self or microbial antigens are being recognized. Indeed, NKT cells have been shown to become activated in several murine models of inflammatory bowel disease (IBD)^{20,21}, a disease characterized by marked inflammation of the gastrointestinal system. In a colitis model induced by the adoptive transfer of naïve CD4⁺ T cells into SCID mice, CD1d was found to be upregulated on colonic epithelial cells.²² In addition, co-transfer of DX5⁺ NKT cells prevented the development of disease in recipient mice, indicating that the activation of NKT cells may be protective in this IBD model. In contrast, a deleterious role for type I NKT cells has been described in oxazolone-induced colitis.²³ Several clinical studies have also reported that human CD1d expression is often either enhanced or reduced in the colon

or cecum of IBD patients, suggesting that changes in CD1d expression levels and subsequent NKT cell dysregulation may play a role in human IBD pathogenesis.^{24,25} Interestingly, a recent study has shown that lamina propria T cells isolated from ulcerative colitis (UC) patients contain increased numbers of CD1d-restricted type II NKT cells.²⁶ Upon stimulation with CD1d-transfectants, these type II NKT cells produce IL-13, which could contribute to the Th2-oriented immune responses observed in UC patients. However, it is not clear whether type II NKT cells are directly involved in the induction of disease or whether type II NKT cells might play a role in other types of IBD.

In this study, we used a type II NKT cell TCR transgenic mouse model (24 $\alpha\beta$ Tg) to study the development and function of type II NKT cells. We found that the level of CD1d expression was critical for proper type II NKT cell development, as we observed significantly reduced type II NKT cell numbers in double transgenic mice (CD1dTg/24 $\alpha\beta$ Tg), which overexpress CD1d. Strikingly, CD1dTg/24 $\alpha\beta$ Tg mice spontaneously develop IBD. Using an adoptive transfer approach, we demonstrated that the type II NKT cells that develop in the context of high levels of CD1d are intrinsically pathogenic and contribute directly to the development of IBD. Interestingly, peripheral exposure of type II NKT cells to high levels of CD1d expression is also sufficient to induce intestinal inflammation. Our results reveal the importance of both CD1d expression levels and type II NKT cell function to IBD pathogenesis.

RESULTS

CD1d expression level affects the negative selection and development of type II NKT cells

24 $\alpha\beta$ Tg mice express a TCR from a CD1d-reactive NKT cell line with V α 3.2 and V β 9 usage. We have previously demonstrated that the development of V α 3.2⁺V β 9⁺ cells (hereafter referred to as 24 $\alpha\beta$ T cells), which exhibit an NKT cell phenotype, is CD1d-dependent.²⁷ Like most type II NKT cells, 24 $\alpha\beta$ T cells can be activated by CD1d-expressing cells in the absence of exogenous antigens.^{28,29} This apparent autoreactivity suggests that the expression levels of CD1d may affect the selection, activation and function of type II NKT cells. We have previously generated *K^b*-CD1dTg mice, in which CD1d expression is driven by the *H-2K^b* MHC class I gene promoter and is therefore highly expressed on a variety of cell types, including thymocytes, antigen presenting cells and intestinal epithelial cells.¹⁵ To determine whether overexpression of CD1d leads to enhanced negative selection of type II NKT cells, we crossed 24 $\alpha\beta$ Tg mice with *K^b*-CD1dTg mice to generate *K^b*-CD1d/24 $\alpha\beta$ double transgenic mice (hereafter referred to as CD1dTg/24 $\alpha\beta$ Tg mice). We found that the percentage and number of 24 $\alpha\beta$ T cells in the spleen of CD1dTg/24 $\alpha\beta$ Tg mice were decreased by approximately 80% when compared to 24 $\alpha\beta$ Tg mice (Figure 1A–C). The percentages of 24 $\alpha\beta$ T cells in the thymus of 24 $\alpha\beta$ Tg and CD1dTg/24 $\alpha\beta$ Tg mice were comparable, yet the total number of thymocytes was decreased by three to five fold in CD1dTg/24 $\alpha\beta$ Tg mice, resulting in a significant decrease in total numbers of 24 $\alpha\beta$ T cells in these mice (Figure 1B and C).

To investigate whether the reduction in 24 $\alpha\beta$ T cell numbers observed in CD1dTg/24 $\alpha\beta$ Tg mice is at least in part due to enhanced negative selection in the thymus, we compared the composition of thymocyte subsets (DP: CD4⁺CD8⁺, CD4^{SP}: CD4⁺CD8[−], CD8^{SP}: CD4[−]CD8⁺, and DN: CD4[−]CD8[−]) in 24 $\alpha\beta$ Tg and CD1dTg/24 $\alpha\beta$ Tg mice. We found that V α 3.2⁺V β 9⁺ DP thymocytes were nearly absent in CD1dTg/24 $\alpha\beta$ Tg mice (Figure 2A and B), suggesting that substantial numbers of 24 $\alpha\beta$ T cells are deleted at the DP stage of development when they encounter CD1d-overexpressing cells. In addition, the remaining DP thymocytes in CD1dTg/24 $\alpha\beta$ Tg mice express higher levels of Nur77, an orphan nuclear steroid receptor involved in thymocyte apoptosis during negative selection³⁰, compared to those in 24 $\alpha\beta$ Tg and 24 $\alpha\beta$ Tg/CD1^{−/−} mice (Figure 2C). Collectively, our data suggest that

an increased avidity to CD1d/self-ligand complexes during thymic development can lead to negative selection of 24 $\alpha\beta$ T cells.

The surface phenotype and functional properties of 24 $\alpha\beta$ T cells are altered in CD1d/24 $\alpha\beta$ Tg mice

To examine whether the remaining 24 $\alpha\beta$ T cells in CD1dTg/24 $\alpha\beta$ Tg mice display a distinct phenotype from those in 24 $\alpha\beta$ Tg mice, we analyzed the expression of various cell surface markers on lymphocytes isolated from the spleen and mesentery lymph nodes (MLN) of 24 $\alpha\beta$ Tg and CD1dTg/24 $\alpha\beta$ Tg mice. We found that surface TCR levels were reduced on 24 $\alpha\beta$ T cells isolated from CD1dTg/24 $\alpha\beta$ Tg mice, compared with cells from 24 $\alpha\beta$ Tg mice (Figure 3A). In addition, the proportion of the CD4⁺ subset of 24 $\alpha\beta$ T cells was significantly lower in CD1dTg/24 $\alpha\beta$ Tg mice (Figure 3B). Further analysis of NK1.1 expression on 24 $\alpha\beta$ T cells revealed a significant increase in the proportion of the CD4⁺NK1.1⁺ subset of 24 $\alpha\beta$ T cells in CD1dTg/24 $\alpha\beta$ Tg mice compared to 24 $\alpha\beta$ Tg mice. In contrast, the CD4⁺NK1.1⁺ subset was reduced by 70–90% in CD1dTg/24 $\alpha\beta$ Tg mice (Figure 3B). The expression patterns of several surface markers, such as Ly6C and Ly49A/D on 24 $\alpha\beta$ T cells were also altered in CD1dTg/24 $\alpha\beta$ Tg mice (data not shown). These results highlight the importance of CD1d expression level on 24 $\alpha\beta$ T cell development.

To assess the function of 24 $\alpha\beta$ T cells from CD1dTg/24 $\alpha\beta$ Tg mice, we examined their cytokine production capacity in response to anti-CD3 stimulation. We found that a significantly higher proportion of 24 $\alpha\beta$ T cells in CD1dTg/24 $\alpha\beta$ Tg mice produced IFN- γ and IL-17A compared to 24 $\alpha\beta$ Tg mice. In contrast, the percentage of 24 $\alpha\beta$ T cells producing Th2-cytokines (IL-4 and IL-13) was significantly reduced in CD1dTg/24 $\alpha\beta$ Tg mice (Figure 3C). These results suggest that the 24 $\alpha\beta$ T cells that develop in the presence of high levels of CD1d have altered functional properties.

CD1dTg/24 $\alpha\beta$ Tg mice spontaneously develop inflammatory bowel disease (IBD)

Although CD1dTg and 24 $\alpha\beta$ Tg single transgenic mice mature normally without any early fatalities, we found that most CD1dTg/24 $\alpha\beta$ Tg mice exhibited symptoms of IBD, including diarrhea, rectal prolapse, and weight loss. The incidence of IBD in CD1dTg/24 $\alpha\beta$ Tg mice progressed with age, reaching approximately 70% by 6 months of age, while less than 10% of 24 $\alpha\beta$ Tg mice developed IBD at this age (Figure 4A). Macroscopically, the colons of CD1dTg/24 $\alpha\beta$ Tg mice were thickened and had lost their normal beaded appearance (Figure 4B). Microscopic analysis of H&E stained colon sections from CD1dTg/24 $\alpha\beta$ Tg mice revealed a severe alteration in mucosal structure, including thickening of epithelial cell layers, loss of goblet cells, a reduction in the number of crypts, and extensive infiltration of leukocytes (Figure 4C). In contrast, colon tissue sections from 24 $\alpha\beta$ Tg mice exhibit relatively normal intestinal architecture (Figure 4C). A histological evaluation of the colon based on three parameters (severity of inflammation, extent of inflammation, and crypt damage) revealed that CD1dTg/24 $\alpha\beta$ Tg mice have a significantly higher clinical score of IBD than do 24 $\alpha\beta$ Tg mice (Figure 4D). Because the inflammation was restricted to rectal and colonic mucosal layers and no intestinal granulomas were detected, the lesions bore a close resemblance to those seen in ulcerative colitis.

To characterize the leukocyte infiltrations in the colon of CD1dTg/24 $\alpha\beta$ Tg mice, we isolated colonic mononuclear cells from 24 $\alpha\beta$ Tg and CD1dTg/24 $\alpha\beta$ Tg mice and analyzed their cellular composition using flow cytometry. We found that while the percentage of 24 $\alpha\beta$ T cells in the colon of CD1dTg/24 $\alpha\beta$ Tg mice was reduced, the percentages of other leukocyte subsets, including B cells, dendritic cells and granulocytes were all increased in CD1dTg/24 $\alpha\beta$ Tg mice compared to 24 $\alpha\beta$ Tg mice (Figure 4E). A functional analysis of colonic mononuclear cells upon anti-CD3 stimulation revealed that a higher proportion of 24 $\alpha\beta$ T

cells in CD1dTg/24 α β Tg mice produce IFN- γ and IL-17A, compared to 24 α β T cells from 24 α β Tg mice (Figure 4F). Taken together, these data demonstrate that increased numbers of type II NKT cells in a setting where CD1d levels are also increased results in dysregulation of immune function and induces chronic inflammation of the colon.

24 α β T cells that develop in the CD1dTg background are pathogenic and can directly cause intestinal inflammation

We observed some discrete V α 3.2⁺V β 9⁻ and V α 3.2⁻V β 9⁺ populations in CD1dTg/24 α β Tg mice, indicating that some endogenous TCR rearrangement occurs during development in these mice. As these populations are relatively prominent in the colon of CD1dTg/24 α β Tg mice (Figure 4E), we crossed both 24 α β Tg and CD1dTg/24 α β Tg mice onto the RAG^{-/-} background to exclude any potential contribution of endogenous T cells to the pathogenesis of IBD. A significant percentage of 24 α β T cells were detected in both 24 α β Tg/RAG^{-/-} and CD1dTg/24 α β Tg/RAG^{-/-} mice and these cells exhibited similar surface phenotypes as their counterparts in a RAG-sufficient background (Figure 5A and 5C). In addition, no V α 3.2⁺V β 9⁻ or V α 3.2⁻V β 9⁺ T cell populations were detectable in these mice. Interestingly, we still observed a high incidence of IBD in CD1dTg/24 α β Tg/RAG^{-/-} mice that steadily reached 50% by 7 months of age (Figure 5B). Furthermore, 24 α β T cells in CD1dTg/24 α β Tg/RAG^{-/-} mice demonstrated a more potent IFN- γ response following *in vitro* stimulation with PMA and ionomycin compared to 24 α β T cells from 24 α β Tg/RAG^{-/-} mice (Figure 5D). This data supports a direct role for 24 α β T cells in the pathogenesis of IBD.

IBD pathogenesis in CD1dTg/24 α β Tg/RAG^{-/-} mice may result from the interaction of developing 24 α β T cells with high levels of CD1d during thymic selection. Alternatively, the disease may be the consequence of peripheral activation of 24 α β T cells upon stimulation with CD1d-overexpressing cells in the periphery, or both mechanisms could play a role. To address this question, we adoptively transferred sorted T cells from 24 α β Tg/RAG^{-/-} or CD1dTg/24 α β Tg/RAG^{-/-} donors into either RAG^{-/-} or CD1dTg/RAG^{-/-} recipient mice. Recipient mice were monitored for the symptoms of IBD and histological scoring of their colon samples was performed 6 weeks after adoptive transfer. As expected, adoptive transfer of T cells from CD1dTg/24 α β Tg/RAG^{-/-} into CD1dTg/RAG^{-/-} mice (group I) recapitulated the robust intestinal inflammation observed in CD1dTg/24 α β Tg/RAG^{-/-} mice (Figure 6A). Interestingly, we observed a significantly higher histological score in RAG^{-/-} recipient mice that had received T cells from CD1dTg/24 α β Tg/RAG^{-/-} mice (group II) compared to mice that had received cells from 24 α β Tg/RAG^{-/-} mice (group IV). This suggests that 24 α β T cells that develop on the CD1dTg background are intrinsically pathogenic. In addition, CD1dTg/RAG^{-/-} recipients that received T cells from 24 α β Tg/RAG^{-/-} mice also developed IBD (group III; Figure 6A and B), suggesting that peripheral exposure to CD1dTg-expressing cells is sufficient for the induction of disease by 24 α β T cells. Collectively, these data suggest that both thymic selection and peripheral activation of 24 α β T cells in an environment where CD1d is highly expressed contribute to dysregulation of 24 α β T cell responses and the development of IBD.

DISCUSSION

In this study, we have defined a role for dysregulated type II NKT cells in the pathogenesis of IBD using a transgenic mouse model that expresses a TCR from an autoreactive type II NKT cell. The use of 24 α β Tg mice has allowed us to directly identify type II NKT cells and examine the effect of CD1d expression level on type II NKT cell regulation, although it has also limited our analysis to a clonal population. However, it is noteworthy that V α 3.2, the V α segment expressed by 24 α β T cells, has been shown to be expressed by a significant proportion of type II NKT cells isolated thus far, suggesting that the type II NKT cell

repertoire is oligoclonal.⁹ Therefore, the information extrapolated from the study of 24 $\alpha\beta$ T cells might be applicable to a sizeable fraction of the type II NKT cells.

Similar to what has been found for type I NKT cells, we found that type II NKT cells undergo negative selection upon encountering high levels of CD1d during development. Nevertheless, some 24 $\alpha\beta$ T cells escape negative selection in CD1dTg/24 $\alpha\beta$ Tg mice. The remaining 24 $\alpha\beta$ T cells in CD1dTg/24 $\alpha\beta$ Tg mice express lower levels of TCR and Ly6C and higher levels of NK1.1, compared to equivalent cells that develop in the B6 background. This suggests that the modulation of signals mediated by these cell surface receptors may be one mechanism by which 24 $\alpha\beta$ T cells escape deletion upon exposure to the high levels of CD1d in CD1dTg/24 $\alpha\beta$ Tg mice. Several studies have shown that high-affinity interactions between autoreactive TCR and self-ligands result in the selection of Treg cells.³¹ Indeed, we consistently observed a higher percentage of Foxp3-expressing cells in CD1dTg/24 $\alpha\beta$ Tg mice compared to 24 $\alpha\beta$ Tg mice (Supplementary Figure 1). The fact that CD1dTg/24 $\alpha\beta$ Tg mice develop IBD suggests that these Foxp3-expressing T cells may not be sufficient to prevent the pathogenic 24 $\alpha\beta$ T cells from inducing IBD in CD1dTg/24 $\alpha\beta$ Tg mice.

The 24 $\alpha\beta$ T cells in CD1dTg/24 $\alpha\beta$ Tg mice produce almost no IL-4 but retain the ability to secrete IFN- γ in response to anti-CD3 stimulation. The impaired IL-4 production in CD1dTg/24 $\alpha\beta$ Tg mice could be due to a lack of IL-4-producing 24 $\alpha\beta$ T cells or to functional anergy of these cells as a consequence of chronic antigen stimulation. To differentiate between these two possibilities, we crossed CD1dTg/24 $\alpha\beta$ Tg mice with 4get reporter mice, in which GFP has been inserted into the *IL-4* locus and can be used to monitor cells with IL-4-producing potential. We found that approximately 20–30% of the 24 $\alpha\beta$ T cells in the thymus and spleen of 24 $\alpha\beta$ Tg/4get mice were GFP⁺, while around 10% of 24 $\alpha\beta$ T cells in CD1dTg/24 $\alpha\beta$ Tg/4get mice were GFP⁺ (Supplementary Figure 2). These results suggest that quantitative differences in the signaling triggered by engagement with CD1d-overexpressing cells during development may either affect the transcriptional activation of the *IL-4* locus in 24 $\alpha\beta$ T cells or lead to the preferential deletion of IL-4-competent 24 $\alpha\beta$ T cells. Besides IL-4, the 24 $\alpha\beta$ T cells in CD1dTg/24 $\alpha\beta$ Tg mice produced lower levels of IL-13, but higher levels of IL-17A and IL-6 in the thymus as well as in the periphery. These findings suggest that interaction with cell types expressing high levels of CD1d during development affects the overall functional capacity of 24 $\alpha\beta$ T cells.

One of the more interesting phenomena that we observed in CD1dTg/24 $\alpha\beta$ Tg mice is the spontaneous development of IBD. The fact that we observed a high incidence of IBD in CD1dTg/24 $\alpha\beta$ Tg mice, but not in single transgenic CD1dTg or 24 $\alpha\beta$ Tg mice indicates that the high expression of CD1d coupled with an increased abundance of type II NKT cells results in the progression of intestinal autoimmune disease. Furthermore, 24 $\alpha\beta$ Tg/CD1^{-/-} mice show no sign of IBD (data not shown), indicating that CD1d is required for type II NKT cell-mediated colitis. Importantly, adoptive transfer of 24 $\alpha\beta$ T cells from CD1dTg/24 $\alpha\beta$ Tg/RAG^{-/-} and 24 $\alpha\beta$ Tg/RAG^{-/-} mice into RAG^{-/-} or CD1dTg/RAG^{-/-} recipients, respectively, led to the development of IBD. These data suggest that interaction with cell types expressing high levels of CD1d either during selection in the thymus or activation in periphery can lead to the dysregulation of type II NKT cell responses and subsequently contribute to the development of IBD. While the CD1d over-expressing cell type(s) responsible for activating 24 $\alpha\beta$ T cells in the gut remain to be identified, our results demonstrate a causative role for dysregulated type II NKT cells in IBD pathogenesis.

We observed high levels of IFN- γ and IL-17A production in the colon of CD1dTg/24 $\alpha\beta$ Tg mice upon anti-CD3 stimulation, but minimal production of Th2-related cytokines (Figure 4F and data not shown). This polarized cytokine response likely contributes to the intestinal inflammation observed in CD1dTg/24 $\alpha\beta$ Tg mice. Our result differs from those of a clinical

study demonstrating an accumulation of IL-13 producing type II NKT cells in the lamina propria of UC patients.²⁶ Several factors may account for this discrepancy: first, the difference in TCR specificity of the reactive NKT cells, and second, the antigen-presenting cell type(s) involved in NKT cell activation, and third, the nature of the microbial stimuli present in the gut of patients and diseased mice. It is likely that type II NKT cells can be skewed towards Th1, Th2 or Th17 responses depending on the strength of the stimulatory signal and the type of antigen presented. In this regard, type I NKT cells in the intestine have been shown to produce different cytokine profiles in response to different stimulatory conditions.^{21,32}

To demonstrate the function of type II NKT cells in another colonic inflammation model, we examined the role of 24 $\alpha\beta$ T cells in dextran sulfate sodium (DSS)-induced colitis. While activation of type I NKT cells has been shown to play a protective role in DSS-induced colitis²⁰, we found that there was a trend toward more severe body weight loss in DSS-treated 24 $\alpha\beta$ Tg mice, compared to DSS-treated wild type B6 mice. Additionally, colon organ cultures of DSS-treated 24 $\alpha\beta$ Tg mice had elevated levels of IFN- γ compared with cultures from similarly treated wild-type mice (Supplementary Figure 3). These data suggest that type II NKT cells may play a pathogenic role in this model. However, the mechanism by which type II NKT cells contribute to the pathogenesis of this IBD model requires further investigation.

The finding that CD1dTg/24 $\alpha\beta$ Tg mice develop an inflammatory response in the colon but not in other organs suggests that the microbial flora in the gut may augment 24 $\alpha\beta$ T cell autoreactivity. Indeed, we found that intestinal inflammation was significantly reduced in CD1dTg/24 $\alpha\beta$ Tg mice that were treated with a broad spectrum of antibiotics compared to untreated mice (Supplementary Figure 4). These results suggest that microbial stimuli may heighten the responses of dysregulated 24 $\alpha\beta$ T cells, resulting in severe intestinal inflammation. Thus, our findings raise the possibility that antibiotic treatment may therapeutically benefit patients whose IBD has been induced by dysfunctional type II NKT cells.

Our studies using CD1dTg/24 $\alpha\beta$ Tg mice provide the first direct evidence of a pathogenic role for type II NKT cells in IBD. Given the functional conservation of CD1d and NKT cells between humans and mice, information obtained from murine studies could shed light on both the development and function of type II NKT cells as well as the role that dysregulated type II NKT cells play in IBD pathogenesis in humans. Our finding that an increased frequency of type II NKT cells in the context of high CD1d expression can lead to development of IBD in mice highlights the importance of tightly regulated CD1d expression. The spontaneous development of colitis in this novel mouse model provides the impetus for examination of CD1d expression levels and type II NKT cell frequency in human IBD patients.

MATERIALS AND METHODS

Mice

CD1^{-/-}, CD1dTg, and 24 $\alpha\beta$ Tg mice have been described previously and were backcrossed for more than ten generations onto a C57BL/6 background.^{15,27,33} 24 $\alpha\beta$ Tg mice were crossed with CD1^{-/-} and CD1dTg mice to generate 24 $\alpha\beta$ Tg/CD1^{-/-} and CD1dTg/24 $\alpha\beta$ Tg mice, respectively. To screen mice for CD1d expression, cells were stained with anti-CD1d mAb 5C6³⁴ and analyzed using flow cytometry. RAG-2^{-/-} mice were purchased from The Jackson Laboratory. All animal work was approved by the Institutional Animal Care and Use Committee.

Cell preparations

Single cell suspensions were prepared from whole tissue by mechanical disruption in HBSS/2% FBS. Hepatic leukocytes were purified through a 36.5% Percoll gradient. Colon tissues were flushed with HBSS/2.5% FBS/1% penicillin/streptomycin, cut into small pieces, and washed with HBSS/1mM DTT and HBSS/10 mM EDTA. Washed colon tissues were digested with 150 µg/ml collagenase and 10 µg/ml DNase at 37 °C for two hours. Colon mononuclear cells were purified by isolating the interface layer from a 40%/75% Percoll gradient.

Antibodies and Flow cytometry

FITC-conjugated anti-Vβ9, Gr1 and TCRβ; PE-conjugated anti-Nur77, NK1.1, CD122, CD44, CD8, Ly6C and B220; APC-conjugated anti-TCRβ and CD11c; Pacific Blue-conjugated anti-CD4, CD11b and B220; biotin-conjugated anti-Vα3.2 were purchased from eBioscience or BD Biosciences. For cell surface staining, cells were pre-incubated with purified 2.4G2 and then stained with the appropriate combinations of mAbs. Nur77 expression on thymocytes was analyzed via intracellular staining using the FoxP3 staining buffer set (eBioscience) with 5 µg/ml anti-Nur77. Flow cytometry was performed with a FACSCanto II and analyzed using FlowJo software.

Intracellular cytokine staining

MLN lymphocytes and colon mononuclear cells were stimulated with plate-bound anti-CD3 (5 µg/ml) for 48 hours, with the addition of PMA (20 ng/ml), ionomycin (1 µM) and monensin (10 µM) during the last 6 hours of culture. For activating hepatic leukocytes, cells were stimulated with PMA and ionomycin for 6 hours, and monensin was added during the last 4 hours of culture. Cells were stained for surface markers, fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and then stained with anti-cytokine antibodies.

Histology

Colon samples were fixed in 10% formalin and laid out in Swiss rolls for paraffin embedding. Paraffin sections were cut and H&E stained to examine the tissue architecture. Histological scores were evaluated in a blind fashion based on the following parameters: (1) severity of inflammation (0, none; 1, mild; 2, moderate; 3, severe), (2) extent of inflammation (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), (3) crypt damage (0, none; 1, basal one-third damaged; 2, basal two-third damaged; 3, crypt lost but surface epithelium present; 4, crypt and surface epithelium lost).³⁵

Adoptive transfer of sorted T cells

Splenocytes were stained with mAb to TCRβ and B220 and TCRβ⁺ B220⁻ cells were sorted via MoFlo. 5×10⁵ to 1×10⁶ sorted T cells (>95% purity) were injected intraperitoneally into recipient mice.

Statistical analysis

Mean values were compared using a Student's *t* test. All statistical analyses were performed using PRISM software. A *P* value of less than 0.05 was considered significant (*, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol.* 2007; 25:297–336. [PubMed: 17150027]
2. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J Exp Med.* 2000; 191:1895–1903. [PubMed: 10839805]
3. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol.* 2004; 4:231–237. [PubMed: 15039760]
4. Exley M, Garcia J, Balk SP, Porcelli S. Requirements for CD1d recognition by human invariant Valpha24⁺ CD4⁺ CD8⁺ T cells. *J Exp Med.* 1997; 186:109–120. [PubMed: 9207002]
5. Cui J, Shin T, Kawano T, Sato H, Kondo E, Taura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science.* 1997; 278:1623–1626. [PubMed: 9374462]
6. Spada FM, Koezuka Y, Porcelli SA. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J Exp Med.* 1998; 188:1529–1534. [PubMed: 9782130]
7. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, Koezuka Y, Kronenberg M. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med.* 2000; 192:741–754. [PubMed: 10974039]
8. Behar SM, Cardell S. Diverse CD1d-restricted T cells: diverse phenotypes, and diverse functions. *Semin Immunol.* 2000; 12:551–560. [PubMed: 11145861]
9. Park SH, Weiss A, Benlagha K, Kyin T, Teyton L, Bendelac A. The mouse CD1d-restricted repertoire is dominated by a few autoreactive T cell receptor families. *J Exp Med.* 2001; 193:893–904. [PubMed: 11304550]
10. Van Rhijn I, Young DC, Im JS, Lavery SB, Illarionov PA, Besra GS, Porcelli SA, Gumperz J, Cheng TY, Moody DB. CD1d-restricted T cell activation by nonlipidic small molecules. *Proc Natl Acad Sci U S A.* 2004; 101:13578–13583. [PubMed: 15342907]
11. Jahng A, Maricic I, Aguilera C, Cardell S, Halder RC, Kumar V. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med.* 2004; 199:947–957. [PubMed: 15051763]
12. Roy KC, Maricic I, Khurana A, Smith TR, Halder RC, Kumar V. Involvement of secretory and endosomal compartments in presentation of an exogenous self-glycolipid to type II NKT cells. *J Immunol.* 2008; 180:2942–2950. [PubMed: 18292516]
13. Exley MA, Tahir SM, Cheng O, Shaulov A, Joyce R, Avigan D, Sackstein R, Balk SP. A major fraction of human bone marrow lymphocytes are Th2-like CD1d-reactive T cells that can suppress mixed lymphocyte responses. *J Immunol.* 2001; 167:5531–5534. [PubMed: 11698421]
14. Terabe M, Berzofsky JA. NKT cells in immunoregulation of tumor immunity: a new immunoregulatory axis. *Trends Immunol.* 2007; 28:491–496. [PubMed: 17964217]
15. Chun T, Page MJ, Gapin L, Matsuda JL, Xu H, Nguyen H, Kang HS, Stanic AK, Joyce S, Koltun WA, Chorney MJ, Kronenberg M, Wang CR. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J Exp Med.* 2003; 197:907–918. [PubMed: 12682110]

16. Pellicci DG, Uldrich AP, Kyparissoudis K, Crowe NY, Brooks AG, Hammond KJ, Sidobre S, Kronenberg M, Smyth MJ, Godfrey DI. Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. *Eur J Immunol.* 2003; 33:1816–1823. [PubMed: 12811841]
17. Zimmer MI, Colmone A, Felio K, Xu H, Ma A, Wang CR. A cell-type specific CD1d expression program modulates invariant NKT cell development and function. *J Immunol.* 2006; 176:1421–1430. [PubMed: 16424169]
18. Skold M, Xiong X, Illarionov PA, Besra GS, Behar SM. Interplay of cytokines and microbial signals in regulation of CD1d expression and NKT cell activation. *J Immunol.* 2005; 175:3584–3593. [PubMed: 16148102]
19. Raghuraman G, Geng Y, Wang CR. IFN-beta-mediated up-regulation of CD1d in bacteria-infected APCs. *J Immunol.* 2006; 177:7841–7848. [PubMed: 17114455]
20. Saubermann LJ, Beck P, De Jong YP, Pitman RS, Ryan MS, Kim HS, Exley M, Snapper S, Balk SP, Hagen SJ, Kanauchi O, Motoki K, Sakai T, Terhorst C, Koezuka Y, Podolsky DK, Blumberg RS. Activation of natural killer T cells by alpha-galactosylceramide in the presence of CD1d provides protection against colitis in mice. *Gastroenterology.* 2000; 119:119–128. [PubMed: 10889161]
21. Ueno Y, Tanaka S, Sumii M, Miyake S, Tazuma S, Taniguchi M, Yamamura T, Chayama K. Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of Valpha14 natural killer T cells in mice. *Inflamm Bowel Dis.* 2005; 11:35–41. [PubMed: 15674111]
22. Hornung M, Farkas SA, Sattler C, Schlitt HJ, Geissler EK. DX5⁺ NKT cells induce the death of colitis-associated cells: involvement of programmed death ligand-1. *Eur J Immunol.* 2006; 36:1210–1221. [PubMed: 16619286]
23. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NKT cells. *Immunity.* 2002; 17:629–638. [PubMed: 12433369]
24. Page MJ, Poritz LS, Tilberg AF, Zhang WJ, Chorney MJ, Koltun WA. Cd1d-restricted cellular lysis by peripheral blood lymphocytes: relevance to the inflammatory bowel diseases. *J Surg Res.* 2000; 92:214–221. [PubMed: 10896824]
25. Perera L, Shao L, Patel A, Evans K, Meresse B, Blumberg R, Geraghty D, Groh V, Spies T, Jabri B, Mayer L. Expression of nonclassical class I molecules by intestinal epithelial cells. *Inflamm Bowel Dis.* 2007; 13:298–307. [PubMed: 17238179]
26. Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, Fichtner-Feigl S, Yang Z, Exley M, Kitani A, Blumberg RS, Mannon P, Strober W. Nonclassical CD1d-restricted NKT cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest.* 2004; 113:1490–1497. [PubMed: 15146247]
27. Skold M, Faizunnessa NN, Wang CR, Cardell S. CD1d-specific NK1. 1⁺ T cells with a transgenic variant TCR. *J Immunol.* 2000; 165:168–174. [PubMed: 10861049]
28. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. CD1-restricted CD4⁺ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med.* 1995; 182:993–1004. [PubMed: 7561702]
29. Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, Koezuka Y, Porcelli SA, Cardell S, Brenner MB, Behar SM. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity.* 2000; 12:211–221. [PubMed: 10714687]
30. Amsen D, Revilla Calvo C, Osborne BA, Kruisbeek AM. Costimulatory signals are required for induction of transcription factor Nur77 during negative selection of CD4⁺CD8⁺ thymocytes. *Proc Natl Acad Sci U S A.* 1999; 96:622–627. [PubMed: 9892683]
31. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Hohenbeck AE, Lerman MA, Naji A, Caton AJ. Thymic selection of CD4⁺CD25⁺ regulatory T cells induced by an agonist self-peptide. *Nat Immunol.* 2001; 2:301–306. [PubMed: 11276200]
32. van Dieren JM, van der Woude CJ, Kuipers EJ, Escher JC, Samsom JN, Blumberg RS, Nieuwenhuis EE. Roles of CD1d-restricted NKT cells in the intestine. *Inflamm Bowel Dis.* 2007; 13:1146–1152. [PubMed: 17476670]

33. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR. Impaired NK1⁺ T cell development and early IL-4 production in CD1-deficient mice. *Immunity*. 1997; 6:459–467. [PubMed: 9133425]
34. Mandal M, Chen XR, Alegre ML, Chiu NM, Chen YH, Castano AR, Wang CR. Tissue distribution, regulation and intracellular localization of murine CD1 molecules. *Mol Immunol*. 1998; 35:525–536. [PubMed: 9809580]
35. Dieleman LA, Palmen MJ, Akol H, Bloemena E, Pena AS, Meuwissen SG, Van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol*. 1998; 114:385–391. [PubMed: 9844047]

Figure 1. CD1d overexpression affects 24αβ T cell development in CD1dTg/24αβTg mice. **(A)** Thymocytes and splenocytes from indicated mice were stained with mAb against Vα3.2 and Vβ9, and analyzed by flow cytometry. Data are representative of 6 experiments. **(B and C)** Bar graphs depict the mean ± SEM of the percentages **(B)** and absolute numbers **(C)** of Vα3.2⁺Vβ9⁺ cells from 24αβTg (n=6) and CD1dTg/24αβTg (n=6) mice.

Figure 2.

Increased CD1d expression leads to enhanced negative selection of 24αβ T cell in CD1dTg/24αβTg mice. (A) Thymocytes from indicated mice were stained with mAb against Vα3.2, Vβ9, CD4 and CD8, and analyzed by flow cytometry. Data shown are representative of 3 experiments. (B) Bar graphs depict the number of DN, DP, CD4^{SP} and CD8^{SP} Vα3.2⁺Vβ9⁺ cells in the thymus of 24αβTg (n=8) and CD1dTg/24αβTg (n=8) mice. (C) Histograms depict the expression of Nur77 on thymocytes (solid line) compared to isotype control staining (filled histogram). Data shown are representative of 2 experiments.

Figure 3.

Altered 24αβ T cell surface phenotype and function observed in CD1dTg/24αβTg mice. **(A)** Flow cytometric analysis of Vα3.2 and Vβ9 expression on splenocytes from 24αβTg and CD1dTg/24αβTg mice. **(B)** Expression of NK1.1, CD4, and CD8 on Vα3.2⁺Vβ9⁺ cells in the spleen and MLN of indicated mice. Data shown are representative of 5 experiments. **(C)** MLN lymphocytes from indicated mice were stimulated with anti-CD3 and intracellularly stained for the indicated cytokines. Data shown are representative of 3 experiments.

Figure 4.

CD1dTg/24αβTg mice develop severe spontaneous inflammation of the large intestine. (A) 24αβTg and CD1dTg/24αβTg mice (n=45 each) were monitored from 3 to 24 weeks of age for symptoms of IBD. (B) Gross observation of representative colon and cecum from 24αβTg and CD1dTg/24αβTg mice (n=10; scale bar=1 cm). (C) H&E stained sections of representative colon samples from indicated mice (200x; scale bar=100 μm). (D) Histological score of colon samples from 24αβTg (n=15) and CD1dTg/24αβTg mice (n=21). (E) Colon mononuclear cells from 24αβTg (n=4) and CD1dTg/24αβTg (n=6) mice were stained with various mAbs to identify 24αβ T cells, non-type II NKT cells (T cells expressing the Tg TCR α or β chain alone), B cells, granulocytes (TCRβ-Gr1^{hi}CD11b⁺) and dendritic cells (TCRβ⁻CD11b⁺CD11c⁺), respectively. Bar graphs depict the mean ± SEM for the proportion of cells within the indicated gate. (F) Colon mononuclear cells from indicated mice were stimulated and intracellularly stained for IFN-γ and IL-17 by Vα3.2⁺Vβ9⁺ cells. Data shown are representative of 3 experiments.

Figure 5.

CD1dTg/24αβTg/RAG^{-/-} mice spontaneously develop IBD. **(A)** Splenocytes and colon mononuclear cells from indicated mice were stained with mAb to Vα3.2 and Vβ9, and analyzed using flow cytometry. Data are representative of 3 experiments. **(B)** 24αβTg/RAG^{-/-} (n=29) and CD1dTg/24αβTg/RAG^{-/-} mice (n=27) were monitored from 4 to 28 weeks of age for signs of IBD. **(C)** Dot plots and histograms depict the expression of indicated markers on Vα3.2⁺Vβ9⁺ cells in the spleen from both genotypes of mice. Data shown are representative of 5 experiments. **(D)** Hepatic leukocytes from 24αβTg/RAG^{-/-} (n=7) and CD1dTg/24αβTg/RAG^{-/-} mice (n=8) were stimulated with PMA/ionomycin and intracellularly stained for IFN-γ production by Vα3.2⁺Vβ9⁺ cells. Data shown are representative of 4 experiments.

Figure 6. Dysregulated type II NKT cell responses directly contribute to intestinal inflammation. **(A)** Sorted T cells from 24αβTg/RAG^{-/-} or CD1dTg/24αβTg/RAG^{-/-} mice were adoptively transferred into either CD1⁺RAG^{-/-} or CD1dTg/RAG^{-/-} recipient mice. H&E stained sections are representative of colons from recipient mice (100x; Scale bar=100 μm). **(B)** Histological scores of colon samples from recipient mice in each experimental group.