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Disparate effects of depletion of CD1d-reactive T cells during early versus late stages of disease in a genetically susceptible model of lupus

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Abstract

Some T cells react with lipid antigens bound to antigen-presenting molecule CD1d. Numbers and functions of a subset of such lipid-reactive T cells are reduced in patients with systemic lupus erythematosus (SLE) and their relatives, as well as in genetically susceptible and chemically induced animal models of lupus-like disease. We have reported that the germline deletion of CD1d exacerbates lupus, suggesting a protective role of these cells in the development of lupus. The use of a knockout mouse model in this study, however, did not allow examination of the role of these cells at different stages of disease. Here, we describe an approach to deplete CD1d-dependent T cells, which allowed us to investigate the role of these cells at different stages of disease in genetically lupus-prone NZB/NZW F1 (BWF1) mice. Repeated intravenous injections of large numbers of CD1d-transfected cells resulted in ~50–75% reduction in these cells, as defined by the expression of CD4, NK1.1 and CD122, and lack of expression of CD62 ligand. TCR $\gamma\delta$ ⁺NK1.1⁺ cells were also reduced in the recipients of CD1d-transfected cells as compared with control recipients. Such depletion of CD1d-reactive T cells in preclinical BWF1 mice resulted in disease acceleration with a significant increase in proteinuria and mortality. In older BWF1 mice having advanced nephritis, however, such depletion of CD1d-reactive T cells resulted in some disease improvement. Taken together, these data as well as our published studies suggest that CD1d-reactive T cells protect against the development of lupus in animal models. However, these cells appear to be unable to suppress established lupus nephritis in these animals, and might even play a disease aggravating role in late stages of disease.

Keywords

animal models; antigen-presenting molecule; autoantibody; lupus nephritis

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Introduction

CD1d molecules are evolutionarily conserved proteins that bind^{1,2} and present^{1,3} lipid antigens to T cells. Such CD1d-reactive T cells usually co-express NK cell and TCR markers, hence are called natural killer T cells. Although small in numbers, these cells can have a profound effect on a variety of immune functions. Importantly, these cells can selectively suppress autoantibody production, while leaving normal antibody production intact.⁴ Several studies have reported a reduction in the numbers of natural killer T cells in peripheral blood of patients with various immune-mediated inflammatory diseases, including scleroderma, type 1 diabetes and systemic lupus erythematosus (SLE) compared with healthy subjects.^{5–11} This natural killer T-cell deficiency was found to correlate with disease activity (SLEDAI) in patients with SLE.⁵ These T cells were restored to normal levels when patients were treated with corticosteroids.¹² Furthermore, the reduced natural killer T-cell numbers in relatives of patients with SLE correlated with the presence of autoimmunity.⁶ Proliferative and cytokine responses of natural killer T cells were also found to be impaired in patients with SLE.^{5,7} These data suggest that natural killer T cells are impaired in patients with SLE, and that impaired natural killer T-cell function might play a role in the pathogenesis of SLE.

Several animal strains, including NZB/NZW F1 (BWF1) and MRL mice, spontaneously develop a T-cell-dependent, autoantibody-mediated disease that mimics SLE.¹³ These animal strains have served as useful models to investigate in vivo mechanisms of pathogenesis of SLE.¹⁴ We have used these animal models to understand the role of CD1d-dependent T cells in the pathogenesis of SLE in vivo. Specifically, we generated BWF1, MRL-lpr and BALB/c mice that are knockout for *Cd1d* gene and determined the effect of loss of CD1d and CD1d-dependent T cells on the development of spontaneous and chemically induced lupus.^{15–17} We found that these animal strains lacking CD1d reactive T cells from birth experience more severe lupus as compared with their CD1d-sufficient littermates.^{15–17} In a reverse set of experiments, we found that treatments with glycolipid antigens ameliorated lupus dermatitis in the MRL-lpr model¹⁸ and lupus nephritis induced by a hydrocarbon oil tetramethyl-pentadecane (TMPD) in BALB/c mice.¹⁹ Contrary to these observations supporting a protective role of CD1d-reactive T cells in lupus, other reports showed that an anti-CD1d antibody prevented lupus,²⁰ whereas treatment in BWF1 mice with established disease using glycolipid antigens that activate CD1d-reactive T cells worsened lupus.²¹ Taken together, these observations raised a possibility that CD1d-reactive T cells might play different roles during different stages of disease. To directly address this issue, we need an approach that could modulate the numbers of these cells at different stages of disease.

In this article, we have taken advantage of the model that natural killer T cells can undergo negative selection when engaged by abundant self-antigen, such as in CD1d-transgenic mice.²² We reasoned that in vivo transfer of large numbers of CD1d-expressing cells might result in depletion of CD1d-reactive T cells, which would allow us to investigate the role of these T cells at different stages of disease development. Our data show that repeated intravenous injections of 50 million CD1d-transfected cells results in more than 50% reduction in CD1d-reactive T cells, which has contrasting consequences for disease development during early versus late stages of lupus nephritis. These findings may have implications for the development of therapies based on CD1d-reactive T cells.

Materials and methods

Mice

Lupus-prone BWF1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed according to approved institutional guidelines.

Cell lines

B-cell lymphoma A20 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in the culture medium, RPMI-1640, containing 10% fetal calf serum, 2-mercaptoethanol, and gentamicin at 37°C in 5% CO₂ incubator. A20 cells were irradiated at 250 Gy, which is known to enhance their antigen-presenting ability²³ and to avoid any direct effect of proliferating A20 cells. Murine CD1d-derived transfected cells were provided by Dr Mitchell Kronenberg.²⁴ Generation of these cells has been described previously.²⁵

Assessment of lupus disease

Survival and renal disease were assessed. Proteinuria was measured on a 0–4+ scale using a colorimetric assay strip for albumin (Albustix, Bayer Co., Elkhart, IN, USA), where 0 = absent, 1+ = 30 (mild), 2+ = 100 (moderate), 3+ = 300 and 4+ = 2000 mg/dl (severe). Severe proteinuria was defined as ≥ 300 mg/dl on two consecutive examinations, as described previously.²⁶

Flow cytometry

Spleen cells were incubated with anti-CD16/32 (2.4G2, PharMingen) to block FcRγII/III, followed by staining with conjugated anti-mouse mAbs (BD PharMingen). FACS analysis was performed using FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA, USA). Dead cells were excluded from the analysis by electronic gating, based on forward and sideways scatter.

Statistical analysis

Frequencies of proteinuria were compared using two-sided Fisher's exact test. Survival analysis was performed using a log-rank test.

Results

In vivo transfer of CD1d-transfected cells in large numbers reduces CD1d reactive T-cell numbers in BWF1 mice

Enhancing CD1d expression in vivo may result in increased presentation of endogenous ligands, leading to positive or negative selection of CD1d-reactive natural killer T cells. In fact, CD1d over-expression in transgenic mice results in reduced numbers of natural killer T cells, implying that these T cells are amenable to negative selection when engaged by abundant self-antigen.²² To determine if transient increases in CD1d expression in adult animals affects natural killer T-cell numbers, we injected CD1d-transfected antigen-presenting cells (APCs) (Figure 1a) in BWF1 mice. In preliminary experiments, animals were injected with varying numbers of CD1d-transfected or untransfected cells (1×10^3 , 1×10^4 , 1×10^5 , 5×10^5 , 5×10^6 , and 5×10^7) intravenously (i.v.), intraperitoneally (i.p.) and intrathymically (i.t.). No effect on NK1.1⁺ T cell numbers was seen at doses below 0.5 million given by all routes. Inconsistent results were seen with i.p. injections. NK1.1⁺ T-cell numbers were slightly (20–40%) reduced in mice injected i.v. or i.t. with 0.5 or 5 million CD1d-transfected cells. Injections i.v. or i.t. of 50 million CD1d-transfected cells led to more than 50% reduction in NK1.1⁺ T cells, although thymic injections resulted in large

variability from animal to animal probably due to technical difficulties. Hence, the i.v. route of 50 million cells was selected for further studies.

As shown in Figure 1b, repeated i.v. injections of 50 million irradiated CD1d-transfected A20 cells resulted in 50–75% reduction in NK1.1⁺ T cells, including CD4⁺NK1.1⁺, NK1.1⁺CD122⁺ or NK1.1⁺CD62L⁺ T cells and NK1.1⁺ TCR $\gamma\delta$ + T cells, when compared with mice injected with the same numbers of untransfected A20 cells (Figure 1b).

In vivo transfer of CD1d-transfected cells in large numbers modulates lupus disease in BWF1 mice

We used such temporal depletion of CD1d reactive T cells to examine their role at different stages of lupus disease development. Young pre-nephritic (12-wk-old) or 22–24-wk-old diseased BWF1 mice were injected with CD1d-transfected cells. Results show that in young mice, injection of high doses (50 million) of CD1d-transfected cells resulted in significant acceleration of severe proteinuria as compared with mice injected with the same numbers of untransfected cells or with saline (Figure 2a). Cumulative mortality was also significantly increased in recipients of CD1d-transfected APCs (Figure 2b).

Intriguingly, transfer of CD1d-transfected APCs at a later age when mice already had moderate proteinuria did not accelerate disease as compared with control recipients. In fact, proteinuria modestly, but significantly, improved in recipients of CD1d-transfected APCs (Figure 2c). Mortality was also reduced in these recipients, but the differences were not statistically significant (Figure 2d).

Discussion

In this article, we show that depletion of CD1d-reactive T cells in early life accelerates disease development in BWF1 mice. These results are in agreement with our previous report showing exacerbation of lupus nephritis in BWF1 mice that are genetically rendered deficient in CD1d.¹⁷ Consistently, in vitro co-culture with NK1.1⁺CD3⁺ cells or their in vivo transfer also reduces anti-DNA Ab production in the B6-*lpr/lpr* model,²⁷ further supporting the regulatory role of natural killer T cells in antibody-mediated autoimmunity. In resonance with these findings, treatment with a glycolipid antigen that activates CD1d-reactive T cells delays the onset of renal disease if given at an early age.³⁵ These observations suggest a protective role of CD1d-reactive T cells during early stages of the development of lupus. In later life of BWF1 mice, however, depletion of CD1d-reactive T cells retards disease, albeit modestly. Consistent with the latter observation, treatment of BWF1 mice with an anti-CD1d mAb has been reported to delay the onset of lupus²⁰ and implantation of liver natural killer T cells under the renal capsule of BWF1 mice has been shown to worsen renal disease.²⁸ These data suggest a possible disease stage-specific role of CD1d-reactive T cells in the development versus progression of lupus disease.

Previous studies have shown that exposure of thymocytes to a glycolipid antigen or increasing CD1d surface expression by transgenesis results in a drastic reduction of CD1d-reactive natural killer T cells.²² Thus, like conventional T cells, CD1d-reactive T cells are susceptible to negative selection. Since i.v. injection of high doses of protein antigens induces deletion of T cells^{29,30} due to supraoptimal surface expression of peptide-MHC class II complexes,³¹ we reasoned that increasing the numbers of CD1d-expressing cells might also result in CD1d-reactive T-cell depletion due to enhanced presentation of endogenous CD1d ligands. Indeed, i.v. or i.t. injections of large numbers of CD1d-transfected cells resulted in a marked reduction in NK1.1⁺ T cells, including CD4⁺NK1.1⁺, NK1.1⁺CD122⁺ or NK1.1⁺CD62L⁺ T cells and NK1.1⁺ TCR $\gamma\delta$ + T cells in adult mice. It is also unclear which of these cell types confer the effects on lupus disease described in this

article. CD1d-reactive natural killer T cells are a diverse group of cells, which likely differ in their functions.^{32,33} For example, some CD1d-reactive T cells that express an invariant TCR (V α 14J α 18 paired with V β 8.2/V β 7/V β 2 in mice and V α 24J α 18 paired with V β 11 in humans), also called classical natural killer T cells or type I natural killer T cells, can be best detected using CD1d tetramers loaded with glycolipids such as α GalCer. Other CD1d-restricted T cells that express diverse TCR α chains, instead of the invariant V α 14J α 18 chain, are termed variant natural killer T cells, also called type II or non-classical natural killer T cells. In addition, some T cells that express natural killer markers NK1.1 and DX5 are not CD1d restricted. Furthermore, some CD1d-reactive T cells respond to glycolipid antigens, whereas others respond to phospholipid antigens, and still other subsets respond to both sets of lipid antigens. Our future studies will determine the effect of CD1d transfectants on these different subsets of CD1d-reactive T cells and will investigate mechanisms whereby in vivo transfer of large numbers of CD1d-expressing cells reduces the numbers of NK1.1⁺ T cells.

It is unclear how depletion of CD1d reactive T cells confers disparate outcomes in early versus late stages of lupus disease in BWF1 mice. Since CD1d-reactive T cells comprise functionally distinct populations of cells,^{33,34} it is possible that some subsets may protect against autoimmunity whereas other subsets might enhance autoimmunity. For example, activation of CD1d-reactive T cells prevents TMPD-induced lupus in BALB/c mice, but augments disease induced in SJL mice. Disease prevention in BALB/c mice requires IL-4 expression, and correlates with Th2 immune responses in the treated animals. Conversely, disease exacerbation in SJL mice correlates with a Th1 bias in CD1d-reactive T cells.¹⁹ Loss or activation of CD1d-reactive T cells may also differentially influence the activation and/or maturation of other cells of the innate and adaptive immune system in early preclinical versus late stages of lupus disease.³⁶ The cytokines produced by activated CD1d-reactive T cells might further stimulate autoreactive T and B cells or dendritic cells in such a way that it perpetuates pathologic autoimmunity in diseased animals that already have markedly activated immune cells.

In summary, our study demonstrates that CD1d-reactive T cells may play different roles in different stages of lupus disease. An understanding of these roles may have implications for the development of novel therapies for the treatment of complex lupus disease. For example, activation of regulatory CD1d-reactive T cells may attenuate early stages of development or flares of lupus nephritis. The idea that CD1d-reactive T cells may suppress lupus disease is particularly appealing, because humans with lupus and related autoimmune diseases have reduced numbers of classical CD1d-reactive natural killer T cells that express the semi-invariant V α 24 TCR.^{5,7,8,10,12} Clearly, a detailed analysis of the different roles of various CD1d-reactive T cell subsets in different stages and manifestations of lupus in patients is warranted to utilize full potential of these cells in therapeutics.

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Figure 1.

Effect of in vivo transfer of CD1d-expressing cells on natural killer T-cell numbers. BWF1 mice received i.v. injections of irradiated CD1d-transfected A20 cells (50 million) twice a week. Control mice received irradiated untransfected 50 million A20 cells. (a) Expression of CD1d on transfected A20 cells. (b) Seven days after the second injection, five mice in each group were sacrificed to harvest spleens. Pooled spleen cells were subjected to red blood cell lysis followed by depletion of I-A^{d+}I-E^{d+} and CD11b⁺ cells using magnetic cell separation. The negatively selected cell fraction consisting of >90% T cells were analyzed for natural killer T cells by flow cytometry. Numbers on dotplots represent the frequency of NK1.1⁺ cells as percentages of T cells.

Figure 2.

Effect of in vivo transfer of CD1d-expressing cells on proteinuria and survival. BWF1 mice were injected i.v. with CD1d-transfected A20 cells (0.5 or 50 million) or saline as indicated in the figure twice a week for 6 weeks ($n=10$ mice per group). Control mice received saline or irradiated untransfected 50 million A20 cells ($n=15$ mice per group). Results are shown severe as % mice with proteinuria (defined as ± 300 mg/dl on two consecutive examinations) and as % cumulative mortality. (a, b) Young (12-week-old), pre-nephritic BWF1 mice. $*p < 0.01$, high-dose transfected cell recipients vs. saline or untransfected cell recipients, log-rank test. (c, d) Diseased, 22–24-week-old BWF1 mice that already had moderate proteinuria. $*p < 0.05$, high-dose transfected cell group vs. other three groups at 29–33 weeks of age, Fisher's exact test. Results are from one representative of three independent experiments.