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Research Article

Invariant NKT Cell Lines Derived from the NOD·H2^{h4} Mouse Enhance Autoimmune Thyroiditis

Rajni B. Sharma,[1] Xiaoguang Fan,[1] Patrizio Caturegli,[1] Noel R. Rose,[1,2] and C. Lynne Burek[1,2]

[1] *Department of Pathology, School of Medicine, Bloomberg School of Public Health, Johns Hopkins Medical Institutions, Johns Hopkins University, Ross Research Building, 648, 720 Rutland Avenue, Baltimore, MD 21205, USA*
[2] *Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins Medical Institutions, Johns Hopkins University, Ross Research Building, 648, 720 Rutland Avenue, Baltimore, MD 21205, USA*

Correspondence should be addressed to C. Lynne Burek, lburek@jhmi.edu

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To study the role of invariant Natural Killer T cell (iNKT) cells in autoimmune thyroiditis, we derived two iNKT cell lines from the spleens of NOD· H2^{h4} mice, a strain that develops spontaneous autoimmune thyroiditis exacerbated by excess dietary iodine. The two lines were CD1d-restricted and expressed CD4$^+$, DX5$^+$, and the Vα4Jα281 gene segment, of the T-cell receptor α locus. Upon stimulation with α-galactosyl-ceramide (α-GalCer), both lines rapidly produced IL-2, IL-4, IFN-γ, IL-10, and TNF-α. Strikingly, a similar cytokine response was also induced by thyroglobulin, one of the most abundant protein in the thyroid gland and a major autoantigen in human autoimmune thyroiditis. Transfer of the iNKT cell lines to syngeneic hosts enhanced autoimmune thyroiditis. Intraperitoneal injections of α-GalCer in iodine primed mice also induced thyroid disease. This paper reports for the first time that iNKT cells respond to thyroglobulin and enhance autoimmune thyroiditis in iodine fed NOD·H2^{h4} mice.

1. Introduction

Autoimmune thyroiditis, also known as Hashimoto's thyroiditis, ranks third in prevalence among the autoimmune disorders in the United States [1], and is determined both by genetic and environmental factors [2]. Some aspects of Hashimoto's thyroiditis can be duplicated using the NOD·H2^{h4} mouse, a strain that spontaneously develops thyroiditis at a low incidence. The incidence and severity of thyroiditis can be exacerbated by supplementation of sodium iodine (NaI) in the drinking water; almost 100% of NOD·H2^{h4} mice drinking NaI-enriched water for 6 to 8 weeks develop moderate to severe thyroiditis [3, 4]. The immunopathology of NOD·H2^{h4} thyroiditis is similar to that of Hashimoto's thyroiditis and is characterized by the development of autoantibodies to thyroglobulin and chronic infiltration of the thyroid gland by mononuclear cells such as CD4$^+$ T cells, CD8$^+$ T cells, B cells, and macrophages [4–6].

Little is known about the role of regulatory immune cells in the pathogenesis of autoimmune thyroiditis. Recent studies have shown that the iNKT cells are a unique population of lymphocytes that downregulate several autoimmune diseases, such as type 1 diabetes and experimental autoimmune encephalomyelitis [7, 8]. Other contrasting studies, however, have shown that iNKT cells may be pathogenic as shown in CD8 T cell-induced NOD diabetes [9] and Con A-induced hepatitis [10, 11]. Thus, since both disease improvement and exacerbation have been shown, even within the same disease model (NOD type I diabetes), the decisive role of iNKT cells in autoimmune pathogenesis remains unclear.

Characteristically iNKT cells share receptors of both T cells and NK cells (NK1.1 and/or DX5) [9, 12] and have an alpha chain of their T-cell receptor encoded by invariant gene segments, Vα14 Jα18 in mice, and Vα24-JQ in humans [13–15]. Two novel antigenic targets for iNKT cells, an exogenous microbial cell antigen and an endogenous lysosomal

glycosphingolipid isoglobotrihexosylceramide (iGb3), have been discovered [16, 17]. Most iNKT cells promptly respond to a synthetic ligand α-galactosyl-ceramide (α-GalCer) and secrete a variety of cytokines characteristic of both Th1 (IFN-γ) and Th2 type (IL-4 and IL-13) responses [18, 19]. iNKT cells interact with target hydrophobic antigens in the context of CD1d, a nonpolymorphic, MHC class I-like molecule usually expressed on conventional antigen presenting cells (APC) [13, 20]. Several studies have documented extensive heterogeneity and diversity in iNKT cell populations that has led to their classification into several subsets [18, 19]. A unique subset of iNKT cells has also been documented that bears CD1d on their surface, lack NK1.1, and autopresent α-GalCer in the absence of conventional APC [21].

Previous studies have shown that adoptive transfer of thyroglobulin-stimulated splenocytes induced autoimmune thyroiditis in mice [22, 23]. However, the precise nature of the specific immune cells that are responsible for transferring disease has not been well delineated. In this paper, we describe two lines of iNKT cells that express surface phenotype of $CD4^+DX5^+$, are CD1d-restricted, autopresent thyroglobulin, and produce both Th1 and Th2 cytokines. We report that these lines of iNKT cells are nonprotective and enhance thyroid autoimmunity in iodine-fed NOD·$H2^{h4}$ mice.

2. Materials and Methods

2.1. Mice. NOD·$H2^{h4}$ mice were bred and maintained in the Johns Hopkins University conventional animal facility. Both male and female mice aged 10 to 12 weeks were used in the studies. Test mice were fed 0.15% of NaI in their drinking water and control mice received regular tap water.

2.2. Purification of Mouse Thyroglobulin. Thyroglobulin was purified as previously described [24]. Briefly, mouse thyroid glands were dissected and homogenized in protease inhibitor buffer. Debris was removed by centrifugation and the supernatant was applied to a 1.6 × 88 cm Sephacryl S300 column (Sigma-Aldrich chemicals, St. Louis, MO) equilibrated with PBS at 4°C. The protein content of each column fraction was determined by spectrophotometry (OD_{280}) and finally by BCA protein assay kit (OD_{560}) (PIERCE, Rockford, IL, USA). Small aliquots of thyroglobulin were collected and frozen at −20°C until used.

2.3. Endotoxin Test. During the thyroglobulin purification process, precautions were taken to avoid any microbial or endotoxin contamination. All thyroglobulin preparations were analyzed using quantitative chromogenic QCL-1000 LAL-test kit bought from Bio-Whittaker, Walkersville, MD, USA. Assay was performed according to the manufacturer's protocol. Briefly, a series of twofold dilutions of endotoxin standards (0.5 EU/mL to 0.008 EU/mL) was prepared. Pyrogen-free, endotoxin-tested water (EU < 0.03, Invitrogen Corporation, Carlsbad California) was used to prepare samples and as a negative control. Serial dilutions of 50 μL of either test or standard samples were prepared and incubated for 10 minutes at 37°C with 100 μL of Limulus amoebolysate, and then with 100 μL of chromogenic substrate for 6 minutes. The reaction was stopped using 100 μL of Stop solution. The absorbance was read spectrophotometrically at 405 nm on ELISA plate reader (Dynatech Laboratories, USA). Thyroglobulin preparations with <0.125 EU were considered endotoxin-free and were used in all assays.

2.4. Thyroglobulin Antibody-Specific ELISA. Purified thyroglobulin was coated onto 96 well Immunolon II plates (Dynatech, USA) at a concentration of 2 μg/mL in carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed 4 times with PBS-Tween 20 (0.05%) and blocked for 2 hrs with 1% BSA-PBS. Plates were then washed 3 times and incubated overnight with mouse sera diluted 1 : 100 in PBS. Mouse thyroglobulin-specific IgG subclasses were detected using appropriate dilutions of secondary antibodies against IgG1 and IgG2b (ICN Inc., Aurora, OH). Color was developed with *p*-nitrophenylphosphate substrate (Sigma, St. Louis, MO). Optical density (OD_{405}) was read on MRX plate reader (Dynatech Laboratories, USA).

2.5. Cell Preparation and Development of Cell Lines. NOD·$H2^{h4}$ mice were fed a low dose of sodium iodide (0.05%) in drinking water for 12 weeks. Spleen mononuclear cells (MNCs) were isolated by density gradient centrifugation on Ficoll Paque (Pharmacia, Biotech, Sweden). Cells were washed and cultured in 24 well plates at a density of 10^6 cells/mL with complete RPMI 1640 supplemented with 10% FBS, 20 IU/mL penicillin, 20 μg/mL streptomycin, 20 mM/L L-glutamine, and 100 μM nonessential amino acids (all from GIBCO BRL, MD, USA). Bone marrow derived dendritic cells were used as feeders. IL-2 (10 ng/mL), IL-4 (100 U/mL), and GM-CSF (50 ng/mL) (PharMingen, San Diego, CA) were added to the cells every 3-4 days, and the cells were stimulated with thyroglobulin every 14–18 days. The cells proliferating in response to thyroglobulin were selected for emergent cell lines and were then developed in 96 well plates at a density of 3×10^{-2} cells/mL. Two lines 1F1 and 2D11 were selected; line 1F1 was derived twice and named as 1F1.1. Bone marrow derived dendritic cells from the same mouse strain were used as feeder layers. Control cell line was derived from an MHC II matched OVA transgenic mouse. $CD4^+$ cells were isolated by magnetic separation using CD4 (L3T4) microbeads (Miltenyi Biotech Inc. Auburn, California USA). Cells were cultured in 6 well plates in supplemented complete medium RPMI 1640 and pulsed with 5 μM chicken $OVA_{323–339}$peptide (ISQAVHAAHAEINEAGR) (Fort Collins, CO USA) using adherent APCs for 24 and 48 hours before adoptive transfer.

2.6. Antibodies and Flow Cytometry. Surface expression of iNKT cells was analyzed using fluorescein isothiocyanate (FITC)/peridinin chlorophyll protein (PerCP) conjugated anti-CD4 (L3T4) and phycoerythrin-(PE-) coupled pan

NK anti-DX5 monoclonal antibodies (mAb). PE-coupled mCD1d mAb (clone 1B1) (BD PharMingen, San Diego, CA, USA) was used to analyze CD1d expression. Intracellular cytokine expression was measured using monoclonal antibodies to mouse IL-2, IL-4, IFN-γ, IL-10, and TNF-α coupled to PE (PharMingen, San Diego, CA). Three-color staining was used to analyze cell surface markers, and intracellular cytokines using standard protocol provided by the manufacturer (BD PharMingen, San Diego, CA, USA). Briefly, developed iNKT cells were stimulated for 4 hours with 45 μg/mL of thyroglobulin in complete RPMI-1640 and incubated for last 2 hours with Golgi stop (BD PharMingen, San Diego, CA, USA). The cells were washed and stained for 30 minutes with cell surface markers followed by cell fixation and staining with intracellular cytokine markers. Flow cytometric analysis was done on a FACSCalibur with Cellquest software (Becton Dickinson, Heidelberg, Germany).

2.7. Expression of Vα14Jα281 in iNKT Cells. Mouse iNKT cells express a TCR$\alpha\beta$ that utilizes invariantly the Vα14 and Jα281 gene segments [15, 25]. Total RNA was extracted from each cell line (RNA easy Mini kit, QIAGEN, Valencia, CA) and reverse transcribed using the primer 5′-TGGCGTTGGTCTCTTT-GAAG-3′, which binds to the constant α region of the TCR. PCR amplification was then performed using the upstream primers 5′-TCCTGGTTGACCAAAAAGAC-3′, which binds to the V α14 region. The 443 bp amplicon was separated on a 2% agarose gel.

2.8. Proliferation Assay. A three-day proliferation assay was performed to test the response of the iNKT cell lines to thyroglobulin. Cells (2×10^4 cells/well) were cultured for 72 hours in complete RPMI-1640 along with adherent peritoneal macrophages as APCs and were stimulated with 45 μg/mL of thyroglobulin. To assess whether the thyroglobulin-specific proliferation of iNKT cells depends on CD1d engagement, proliferation assays were performed with different concentrations of purified mouse anti-CD1d mAb for blocking (3.0–0.19 μg/well, 0 representing no mAb). Cells were pulsed with 1 μCi of [methyl-3H] thymidine (Amersham, Pharmacia, NJ, USA) for the last 18 hours. Proliferation was measured as incorporated [methyl-^{3}H] thymidine on a matrix 96 direct β-scintillation counter (Wallace, Germany). Data represents mean values of triplicate wells after 4 minutes of counts on the beta-counter.

2.9. CD1d Tetramer Staining. Recombinant soluble CD1d protein (gift from Dr. A. Bendelac) was incubated with thyroglobulin (100 ng) or with a 2 μM solution of αGalCer in 0.005% Tween 20; unbound protein was removed by centrifugation dialysis in a Microcon YM-30 tube (Millipore, Bedford, MA). Tetramers were formed by mixing thyroglobulin or αGalCer-loaded monomers with PE-conjugated streptavidin (at 5 : 1). Staining was done by incubating cells on ice for 3 h with tetramers at a concentration equivalent to 3–5 μg/mL of CD1d1. For the IL-2 assay NKT hybridoma was similarly incubated with loaded CD1d and secretory IL-2 was measured in the supernatant.

2.10. Adoptive Transfer. iNKT cell lines were incubated with thyroglobulin for 4 hrs at 37°C in a humidified, 5% CO_2 incubator prior to transfer into 10-to-12 week-old NOD$\cdot$H2^{h4} mice. The cells were washed twice and resuspended in sterile PBS and injected intravenously (i.v.) on days 0 and 2 at a concentration of 5×10^6 cells/mouse. All mice were fed 0.15% NaI in their drinking water 2 weeks prior to cell transfer. Mice were sacrificed 14 days postinjection; thyroids were dissected, and sera collected for detection of autoantibodies to thyroglobulin. Control groups received iodine water and injections of sterile PBS i.v. at same time as test mice instead of cell transfers. As a control cell line, OVA-specific CD4$^+$ cells were similarly transferred to iodine fed and age-matched mice after stimulation with 5 μM chicken OVA$_{323–339}$ peptide (ISQAVHAAHAEINEAGR) (Fort Collins, CO USA) using adherent APCs for 24 or 48 hours before adoptive transfer.

2.11. Tracking of Transferred Cells. In order to track these cells, iNKT cell lines which were derived from Thy1.2 NOD$\cdot$H2^{h4} mice were adoptively transferred to iodine fed Thy1.1 NOD$\cdot$H2^{h4} mice at concentrations described above. Cells were isolated from the thyroids after 14 days posttransfers and were analyzed after enzymatic digestion by flow cytometry as previously described [26].

2.12. In Vivo α-GalCer Treatment and Iodine Priming of Mice. NOD$\cdot$H2^{h4} mice were used: experimental group $n = 9$ and control groups of Vehicle alone $n = 5$ and iodine alone group $n = 7$. Both males and females were included in the study. Iodine was supplemented in their drinking water for two weeks prior to α-GalCer injections. Two α-GalCer i.p. injections of 100 μg/mouse on days 0 & 7 were given. Mice were sacrificed on day 14.

2.13. Tissue and Serum Collection. Sera were collected prior to iodine water treatment (day-14), on the day of adoptive transfer of cells (day-0), and at day 14 posttransfer. Thyroids were fixed in 10% buffered formalin for 2 days, and submitted for histological staining. Blood samples were incubated at room temperature for 30 minutes; sera were collected after centrifugation and stored at −80°C until used.

2.14. Histology. Paraffin embedded sections of thyroids were stained with hematoxylin and eosin and graded from 0 to 4 score based on the extent of mononuclear cell infiltration. A grade of 0 was assigned for no lesions, 1 for <20% infiltration, 2 for 20–30%, 3 for >30–50%, 4 for >50% infiltration of the thyroid.

2.15. Statistics. All comparisons of normally distributed data were made using Student's *t*-test; otherwise, Mann-Whitney

U test was used. Test values were considered to be statistically significant from control values at $P < .05$.

3. Results

3.1. Adoptive Transfer of Cell Lines Resulted in Autoimmune Thyroiditis. Two iNKT cell lines were derived from the spleen cells of NOD·H2^{h4} mice stimulated with thyroglobulin as described in methods. The possible role of these cell lines in autoimmune thyroiditis was first determined. Adoptive transfers were performed with both iNKT cell lines along with appropriate control cells such as OVA-specific CD4+ cells. Adoptive transfer experiments with both cell lines were performed in iodine pretreated NOD·H2^{h4} mice. Mice were sacrificed at day 14 following adoptive transfer, and results were analyzed by (i) scoring thyroid histopathology, and (ii) assessing thyroglobulin antibody by ELISA.

3.1.1. Thyroid Histology Showed Increased Cellular Infiltration. Histological analysis of the cellular infiltrates of mice receiving either cell line 1F1.1 or 2D11 cells revealed moderate to dense cellular infiltration scoring from 2-3 (30–50%) as well as intense follicular destruction as compared to controls (Figures 1(a) and 1(b)). Table 1 shows a summary of results of disease frequency and severity of lesions developed postadoptive transfer. Two control groups were used; one group received iodine but no cell transfer and other did not receive iodine but did receive equivalent number of cells as the experimental groups. The control group that received NaI in their drinking water for same time period as the experimental group did not develop lesions in the thyroid except for one mouse that developed a low level of thyroiditis, probably due to the spontaneous phenotype of the mouse model. The adoptive transfer of line 1F1.1 resulted in development of lesion scores from 1–3 in 8 of 12 mice. Similarly line 2D11 resulted in lesion score of 1-2 in all 4 of 4 mice (Table 1). Adoptive transfer of control OVA-specific CD4$^+$ cells showed no infiltration of the thyroid glands in any of the mice (Table 1).

3.1.2. Thyroglobulin Antibody Levels Increased after Adoptive Transfer of NKT Cells. Thyroglobulin-specific IgG1 and IgG2b autoantibody subclasses were detected in the serum of iNKT cell transfer recipients. Figure 2 shows results from a representative adoptive transfer experiment from line 1F1.1. Significantly increased levels of IgG1 (Figures 2(a) and 2(b)) ($P < .005$) and IgG2b antibodies (Figures 2(c) and 2(d)) ($P = .02$) to thyroglobulin were seen in almost all of the mice receiving transfers in comparison to control mice that received NaI alone (Figure 2). Since the production of autoantibodies to thyroglobulin is indicative of thyroid autoimmunity, these results suggested that all of the mice receiving 1F1.1 cells in this particular experiment ($n = 9$) developed enhanced response to thyroid autoantigens culminating in thyroiditis. None of the mice that received control OVA-specific CD4$^+$ cells developed antibody to thyroglobulin (data not shown). Since we now knew that our cell lines could induce autoimmune thyroiditis in NaI-treated NOD·H2^{h4} mice, we proceeded to characterize these cells in detail.

3.2. Proliferative Response of Cell Lines to Mouse Thyroglobulin. To show that the cell lines respond to thyroglobulin, we performed an *in vitro* proliferation assay. The two cell lines, 1F1.1 and 2D11, were cultured for 72 hours at a cell concentration of 2×10^4/well on irradiated adherent peritoneal macrophages with 45 μg/mL of thyroglobulin. The same cell lines were cultured with either ovalbumin or medium alone as controls. Both cell lines showed a significantly higher proliferation in response to thyroglobulin ($P = 7.9499\text{E}-05$); however, both lines also showed a weak response to ovalbumin (Figure 3). Thus, we hypothesized that iNKT cells that are strongly responsive to our thyroglobulin preparation enhance thyroid autoimmunity and contribute to disease.

3.3. Intracellular Cytokine Profiles of Cell Lines. iNKT cells promptly produce various cytokines in response to α-GalCer. To determine the intracytoplasmic cytokine expression, the two lines were stimulated with thyroglobulin or α-GalCer. Intracellular immunofluorescent staining was performed at various time points (2, 4, 8, 18, 24, and 48 hours), to determine the optimal time-point for the intracellular expression of cytokines from the two lines (data not shown). After performing the kinetics, we found that both lines expressed intracellular cytokines within 4 hours of stimulation. The expression of most cytokines disappeared after 10 hours. Therefore, the 4-hour time point was used for further assays and analyses. Interestingly, both cell lines displayed different patterns of intracellular cytokine production (Figures 4(a) and 4(b)). Although both cell lines had high numbers of IL-2-producing cells initially, but upon long-term culture they lost this ability of IL-2 secretion. Line 1F1.1 showed a polarization towards Th1 response. High number of IFN-γ producing cells (approximately 72–82% with thyroglobulin or α-GalCer resp.) and few IL-4 secreting cells (~2%) were recorded as shown in Figure 4(a). Line 2D11 showed moderate numbers of both, IFN-γ (approximately 50–54% with thyroglobulin or α-GalCer resp.) and IL-4 (approximately 28–44% with thyroglobulin or α-GalCer respectively) producing cells (Figure 4(b)). Thus, line 2D11 showed a different cytokine profile as compared to line 1F1.1. IL-10 was found in significant proportions of cells in both the lines with 25–70% in 1F1.1 (with thyroglobulin or α-GalCer resp.) and 38–42% in line 2D11 (with thyroglobulin or α-GalCer, resp.). TNF-α was found in almost all the cells of line 1F1.1 but only 30–35% cells of line 2D11. Thus, although the variation in the numbers of cytokines secreting cells existed among the two lines, the pattern of cytokines within the lines in response to either thyroglobulin or α-GalCer was similar. Furthermore, upon repeated stimulation, the percentages of cytokine producing cells of a particular line remained constant (Figure 4 represents data from one representative experiment).

3.4. Characterization of the Phenotype of Cell Lines. iNKT cell lines that proliferated in response to thyroglobulin and

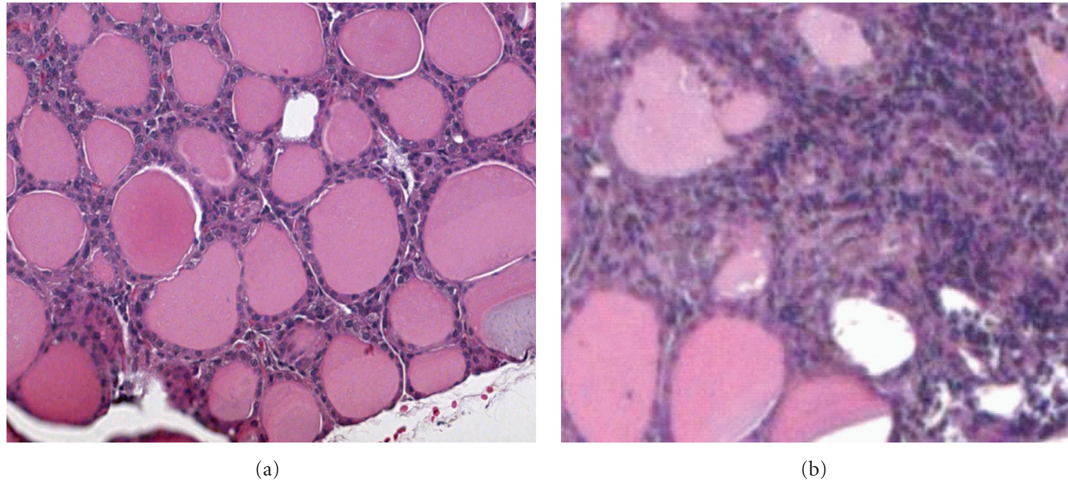


Figure 1: A representative figure of thyroid gland histology from a control mouse and a adaptively transferred with NKT cell line 1F1.1 is shown after hematoxylin and eosin (H & E) staining. (a) Normal thyroid histology showing follicles surrounded with thyrocytes. (b) Section from thyroid gland after 14 days after adoptive transfer of iNKT cells line 1F1.1. Cellular infiltration and disruption of normal thyroid histology was observed. The thyroid histology was assessed as a 5-point score as described in Table 1.

Table 1: Incidence and severity of thyroiditis after transfer of iNKT cell clones to NOD·H2^{h4} mice.

2-week administration of NaI	Transferred iNKT cell clone	Thyroiditis incidence	Thyroiditis severity*				
			0	1	2	3	4
Yes	—	1/12	11	1	0	0	0
No	1F1.1	0/4	4	0	0	0	0
Yes	1F1.1	8/12	4	3	4	1	0
Yes	2D11	4/4	0	1	3	0	0
Yes	CD4$^+$ (OVA specific)	0/4	4	0	0	0	0
Yes	*α-GalCer*	5/9	4	1	3	0	1
Yes	*Vehicle*	1/7	6	1	0	0	0

* Thyroiditis severity was scored as follows: 0 for no lesions, 1 for <20% infiltration, 2 for 20–30%, 3 for >30–50%, and 4 for >50% infiltration of the thyroid.

produced both Th1 and Th2 type cytokines were characterized for the expression of various cell phenotypic markers. The cells were stained for the characteristic surface markers associated with T cells (TCR$\alpha\beta$, CD4, and CD3) and NK cells (DX5, a pan-NK cell marker). Unstimulated cell lines were also stained to determine the constitutive expression of various surface markers. Both the cell lines in resting as well as stimulated states expressed surface markers for TCR$\alpha\beta^+$, CD4$^+$, CD3$^+$, DX5$^+$, and CD69$^+$ as shown in Table 2. In addition to common iNKT cell markers, our iNKT cells also expressed CD1d on their surface. In order to detect whether macrophages could be present in the cell cultures, accounting for CD1d expression, macrophage/dendritic cell markers (Mac1, CD80, and CD86) were also tested. None of the cell lines showed detectable levels of such markers for macrophages, dendritic cells, or other populations such as CD8$^+$ or B220$^+$. However, coexpression of CD4$^+$ and DX5$^+$ was detected on 95–99% cells of both cell lines (Table 2, Figure 5). It is not surprising that NK1.1, a common marker for NK cells, was not observed on the cell lines since NK cells from parental NOD mice do not display this allelic marker [12].

Although these results suggested that the lines are indeed a subset of iNKT cells, further confirmation was required. A classical characteristic of most iNKT cells is their restricted usage of the invariant chain TCR$\alpha\beta$ encoded by Vα14Jα281 gene rearrangement [14, 15]. Importantly, both cell lines expressed Vα14Jα281 as shown by RT-PCR (Figure 6). In contrast, a CD4$^+$ NK1.1$^-$/DX5$^-$ T cell clone, used as a negative control, did not show any such expression (Figure 6). These results confirm that the cell lines produced and stimulated by our thyroglobulin preparation derived from NOD·H2^{h4} mice are a subset of iNKT cells. Hence, the mixed Th1/Th2 cytokine profile from these cells as shown above is not surprising, since autoimmune thyroiditis shows both Th1 and Th2 cytokines with disease pathology [5, 27], and iNKT cells are also known to release large amounts of both types of cytokines following four hours of stimulation.

3.5. CD1d-Restriction of iNKT Cell Lines. Most iNKT cell subsets recognize lipids or hydrophobic peptide antigens in the context of CD1d, that is usually expressed on antigen presenting cells [28]. We found that our iNKT cell lines

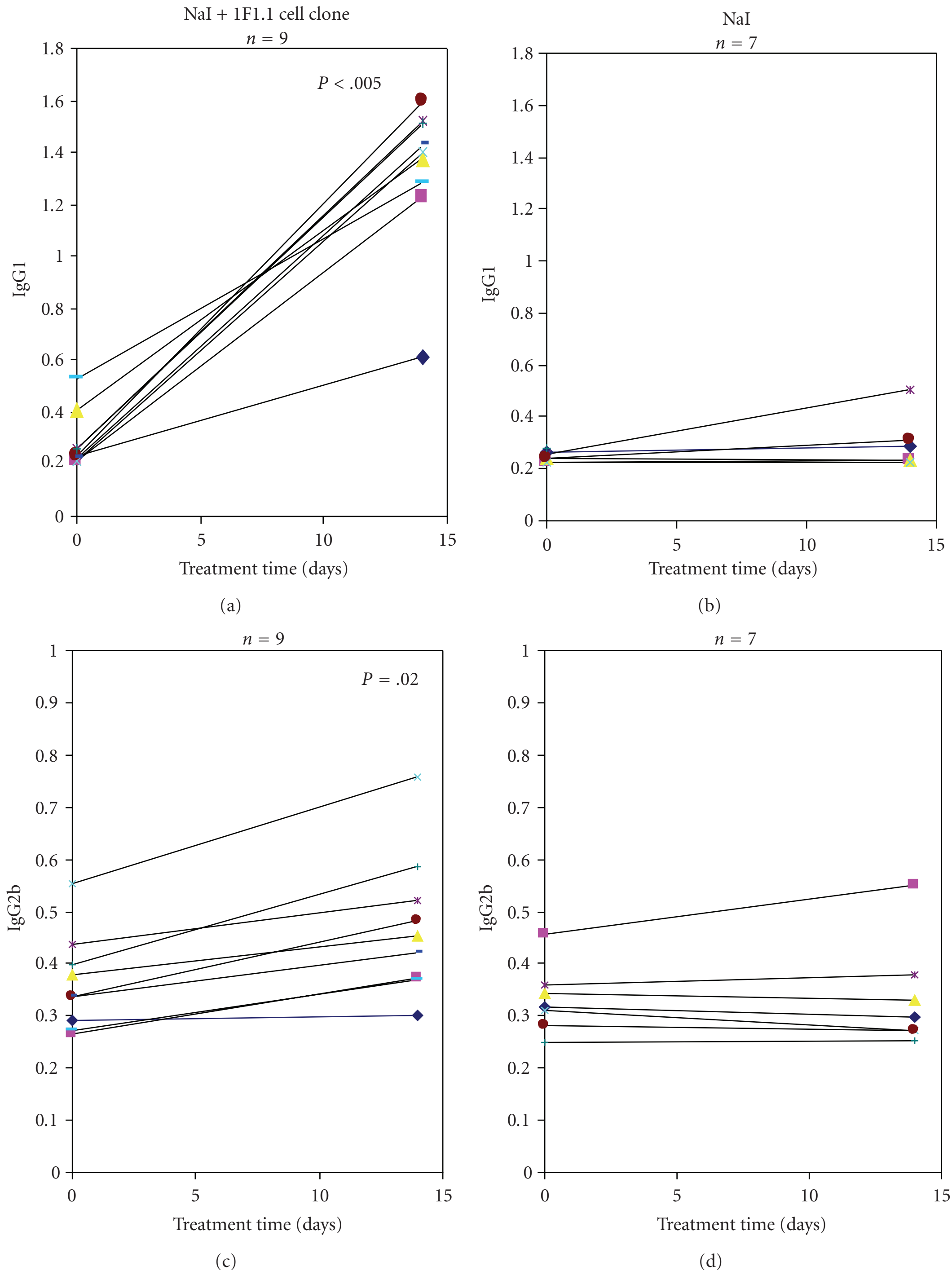
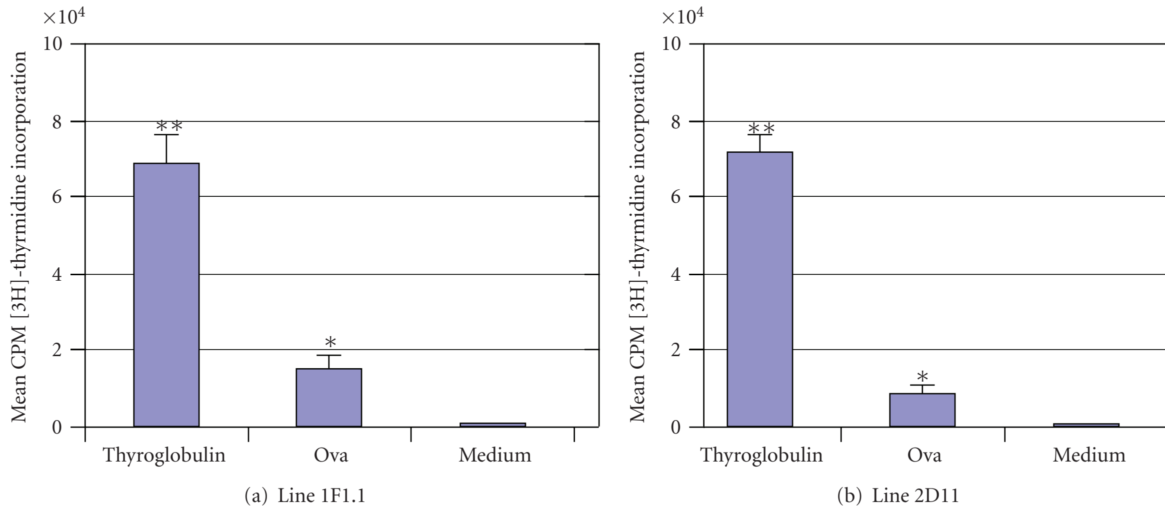


Figure 2: Adoptive transfer of iNKT line 1F1.1 in 8–10-week-old syngeneic mice induced antibodies to thyroglobulin. Mice in panels (a) and (c) received pretreatment of iodine and received iNKT cells. Both, IgG1 and IgG2b (a and c) antibody titers in the treatment groups (posttransfer day 14) were significantly higher as compared to the control group of mice (b and d). Shown IgG1 with $P < .005$ and IgG2b with $P = .02$. Control group mice on panels (b) and (d) received iodine pretreatment but no cells. No significant increase in the antibody titer to thyroglobulin was seen in control groups.

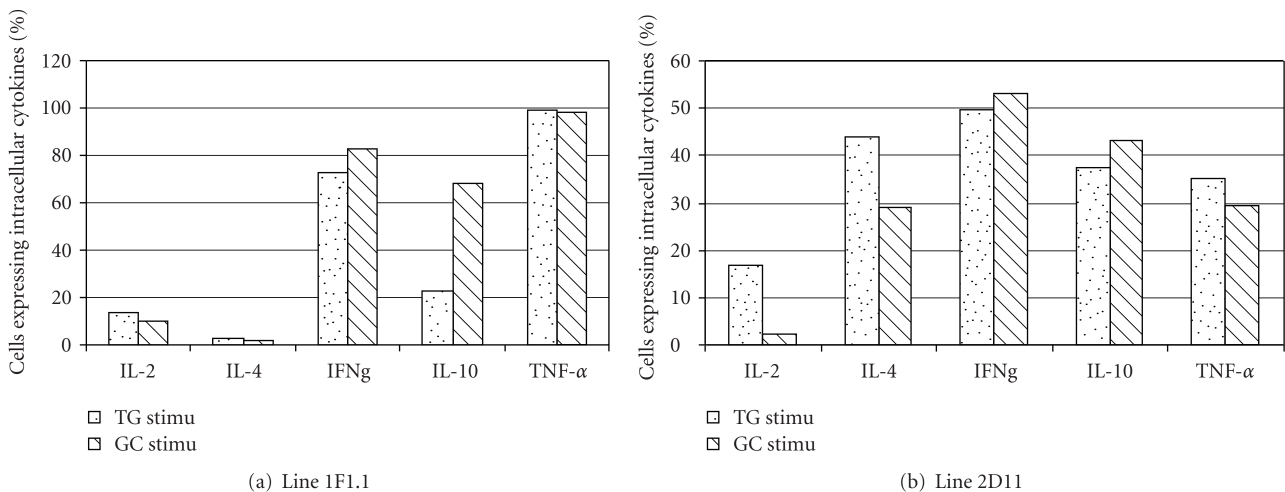
expressed CD1d and proliferated in response to thyroglobulin in the absence of conventional APCs (Figure 7). The proliferation assay results suggested that CD1d bearing iNKT cells are capable of auto-presenting antigen in absence of conventional APCs as described earlier by Hameg et al. [21]. To test the dependence of the cell lines on CD1d for stimulation by thyroglobulin, we blocked CD1d using a CD1d monoclonal antibody (mAb). We found that thyroglobulin-specific proliferation was completely abrogated in a dose-dependent fashion with CD1d mAb treatment, whereas



(a) Line 1F1.1

(b) Line 2D11

FIGURE 3: *In vitro* response of iNKT cells to thyroglobulin. A 72-hour proliferation assay was performed in response to 45 μg/mL of thyroglobulin. [3H]-thymidine incorporation was used as an indicator of stimulation response. Both cell lines showed a significantly higher proliferation in response to thyroglobulin. A few cells also proliferated in response to Ovalbumin (Ova). Results are expressed as mean counts/4 minutes of [3H]-thyrmidine uptake. (Representative of 3 independent experiments).



(a) Line 1F1.1

(b) Line 2D11

FIGURE 4: Cytokine response of two iNKT cell lines. Cells were stimulated with thyroglobulin or α-GalCer four hours prior to staining. Intracellular cytokine expressions were then detected by flow cytometry as described in methods. Both lines showed a burst of cytokines after 4 hours of stimulation. (a) Line 1F1.1 showing high levels IFN-γ, but low levels of IL-4. (b) Line 2D11 showing elevated levels of both IFN-γ and IL-4. Data represents percent of total iNKT cells expressing intracellular cytokines.

unblocked cells efficiently proliferated in response to thyroglobulin stimulation (Figure 7).

To confirm the CD1d specificity of iNKT cell lines, we used tetramers for CD1d. FACS staining using α-GalCer-CD1d-specific tetramers confirmed that the lines are iNKT cells. Clones cells gated on $CD4^+DX5^+$ were found to be >88% positive for CD1d tetramer staining (Figures 8(a) and 8(b)). These results verify that our cell lines are functionally CD1d-restricted and recognized α-GalCer like typical iNKT cell clones.

3.6. In Vivo Treatment of α-GalCer Enhanced Thyroiditis in NOD·H2^{h4} Mice. Mice were given two α-GalCer injections i.p. after a short period (two weeks) of iodine treatment. As shown in Table 1, 55% of mice that received α-GalCer injections developed infiltration of the thyroid gland after 14 days. Approximately 22% of mice (2 of 9) also developed autoantibody to thyroglobulin (data not shown). From the control group, only 14% of mice (1 of 7) developed low-grade thyroid histology, but none of them developed detectable levels of thyroid autoantibody (Table 1).

3.7. Tracking iNKT Cells in the Thyroids. Since iNKT cells contribute to autoimmune thyroid autoimmunity in transfer experiments, it is important to determine the site of action of their adoptive transfer. To address this question

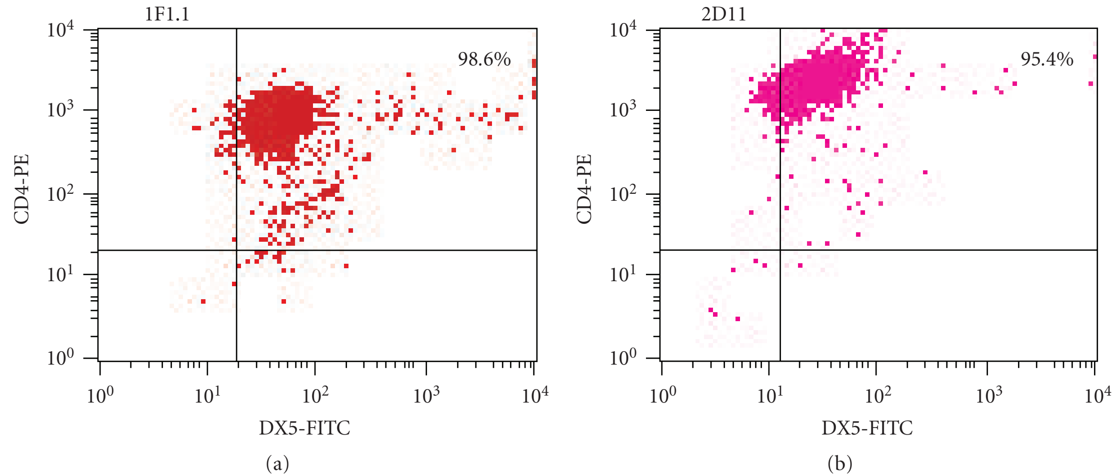


FIGURE 5: Surface phenotypic expression of two iNKT cell lines by two-color flow cytometry. Cells stained with mouse monoclonal antibody CD4-PE and DX5-FITC were analyzed after a side scatter versus forward scatter gate on live lymphocytes. Both iNKT clones showed a double-positive expression $CD4^+DX5^+$ on their surface. Line 1F1.1 showed a pure population of >98% and line 2D11 >95%.

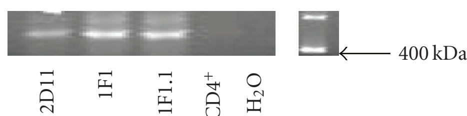


FIGURE 6: Expression of Vα14 Jα281 on iNKT cells. Mouse iNKT cells express a TCR$\alpha\beta$ that utilizes invariant Vα14 and Jα281 gene segments. RT-PCR using specific primers showed that lines 1F1, 1F1.1 subderived line of 1F1 and 2D11, all expressed Vα14Jα281 region, whereas a standard $CD4^+$ T cell clone was negative for this expression. The 443 bp amplicon was separated on a 2% agarose gel.

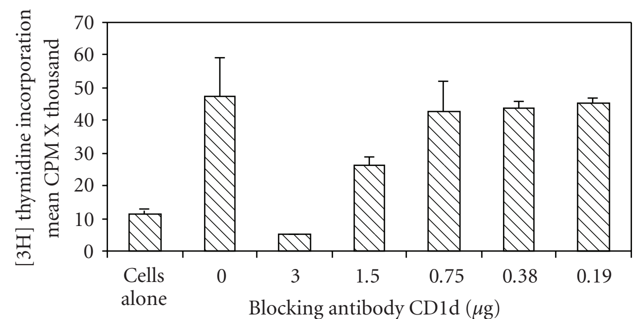


FIGURE 7: Demonstration of CD1d dependence of iNKT cells. Proliferation of line 1F1.1 in response to thyroglobulin was assessed after blocking CD1d. Different concentrations of mouse CD1d mAb were used starting from 0.0–3.0 μg/well. Concentration 0 represents no blocking. Proliferation in response to thyroglobulin was abrogated after CD1d blocking showing that iNKT cells recognized thyroglobulin in a CD1d-dependent manner. [3H] thymidine incorporation was measured as an indicator of proliferative response. Data represents mean CPM of triplicate wells from each concentration.

we performed adoptive transfer experiments using iNKT cell lines from NOD$\cdot$H2^{h4} mice with Thy1.2 expression into NOD$\cdot$H2^{h4} mice expressing Thy1.1. The cells were transferred in a similar manner as described earlier. Thyroids were collected for disease assessment 14 days following cell transfers. Single-cell suspensions were prepared and analyzed for detection of Thy1.2 iNKT cells. No infiltrating Thy1.2 expressing iNKT cells were detected by flow cytometry analysis (data not shown). However, we could detect $CD45^+$ infiltrating lymphocytes on day 14, indicating disease progression. We interpret these results to show that (i) these cells were short lived and/or (ii) influenced disease development indirectly through their cytokines most probably in the local lymph nodes but not intrusive in the thyroid gland itself.

4. Discussion

In this paper, we report that adoptive transfer of iNKT cell lines enhanced spontaneous thyroiditis in susceptible recipient mice. The characterization of the cell lines was established initially by detecting the surface phenotype of both $CD4^+$ and $DX5^+$ (a pan-NK cell marker), and then by the expression of invariant TCR α-chain: Vα14Jα281. Both cell lines were found to coexpress $CD4^+$ and $DX5^+$ on their surface. NK1.1, a marker commonly used to identify NK cells, was not detected on either of cell lines. This is not surprising since NOD mice, along with many other mouse strains do not express the NK1.1 antigen [12]. The expression of DX5 protein has been shown to be positive for all mouse strains studied and hence has been widely used as a marker to identify NK cells [9, 13]. The overall phenotypic analysis of the cell lines indicated that the vast majority of cells within the culture, at least 95–99% of the cells, expressed the iNKT cell phenotype.

In order to gain a better understanding of the functional characteristics of the iNKT cell lines, we examined their response to thyroglobulin or ovalbumin stimulation. In addition to proliferation in response to thyroglobulin, both lines responded weakly to ovalbumin. CD1d is known to have a much deeper groove than the classical MHC

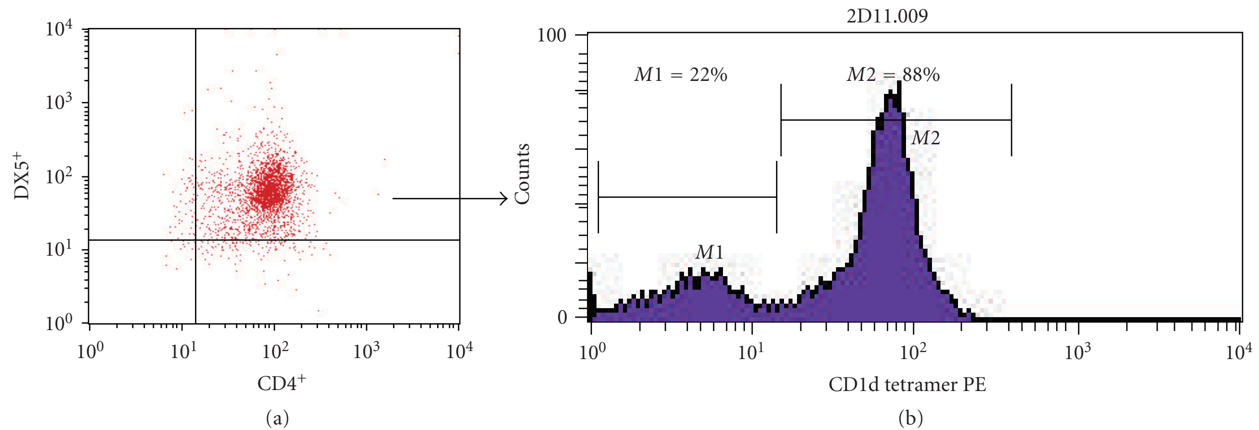


Figure 8: CD1d tetramer staining to show CD1d-restriction of iNKT cells. Flow cytometry was used to determine the percentage of tetramer positive cells from the homogeneous population of $CD4^+DX5^+$ iNKT cells. (a) Surface phenotype showing $CD4^+DX5^+$ of line 2D11. (b) Tetramer positive cells represented under M2 gate showing 88.8 % CD1d tetramer positive cells.

molecules, which binds with a high affinity to glycolipids and hydrophobic peptides [28]. It is known that the hydrophobic end of ovalbumin also binds to the CD1d groove with high affinity. Perhaps thyroglobulin, having many hydrophobic areas, is similarly presented by CD1d to iNKT cells [29]. Even though subsets of iNKT cells recognize antigens presented by CD1d, little is known about the role of exogenous hydrophobic peptide antigens, such as thyroglobulin or other natural ligands, in the processing, presentation, selection, and development of iNKT cells. Our proliferation data suggest that thyroglobulin, or a derived peptide, may be a candidate ligand for CD1d-dependent iNKT cell stimulation in iodine-fed NOD$\cdot$H2^{h4} mice. Iodine modification may still further contribute to the hydrophobic nature and stability of the thyroid autoantigen [30, 31].

The presence of CD1d on the surface of the iNKT cell lines suggests that these cells may be able to present thyroglobulin in an autocrine or paracrine manner. Because CD1d blocking inhibited the stimulation of these cells, it confirmed their CD1d restriction. However, it is not yet clear how thyroglobulin is processed. A study on characterization and sequence of human thyroglobulin (hTg) recognized the disease-inducing effect of a 40-amino acid (F40D) peptide from hTg. The pathogenic F40D peptide of human thyroglobulin was found to be highly hydrophobic in nature and located at the end of the second third of the thyroglobulin molecule. Injection of this peptide into thyroiditis-susceptible CBA/J mice strain induced severe autoimmune thyroiditis [32]. It is a possibility that iNKT cells may recognize hydrophobic peptide F40D or a similar hydrophobic peptide, leading to pathogenicity in NOD$\cdot$H2^{h4} similar to CBA/J. Alternatively iNKT cells may autopresent antigen by a mechanism similar to a subset of $CD8^+$ T cells that autopresents α-GalCer [21]. Recent studies demonstrate that human CD1d restricted T cells via $\alpha\beta$TCR, under certain inflammatory and autoimmune conditions, are capable of recognizing molecular structures of nonlipid small peptide molecules [33]. It appears that processing of antigen may take place by more than one way in vivo depending on the nature of antigen resulting in generation of pathogenic immune responses against more than one epitope of thyroglobulin. A recent study has shown that a plasminogen-like protein that is present in the apical region of thyroid epithelial cells naturally degrades thyroglobulin in order to maintain the concentration of thyroglobulin in the lumen of thyrocytes [34]. We speculate that during this process of degradation small hydrophobic antigenic fragments are formed that could be presented to iNKT cells in context of CD1d. Therefore, studying the factors promoting pathogenic epitopes during the processing and presentation of thyroglobulin by CD1d-bearing APCs should help to learn more about the recognition of thyroglobulin by iNKT cells and their role in disease pathogenesis.

The unique capacity of iNKT cells to promptly release cytokines upon antigenic stimulation is thought to be the basis of their regulatory functions during the effector phase of the immune response, especially in regulation of autoimmune disorders [8, 35]. iNKT cells may downregulate disease either by secreting cytokines that are protective [7] or by recruiting tolerogenic dendritic cells [36]. FACS analysis of the intracellular cytokine profiles of our iNKT cell lines revealed a diverse cytokine profile representing both Th1 and Th2 types after 4 hours of thyroglobulin stimulation (Figure 4) as supported by previous studies from iNKT cells stimulated *in vitro* [37, 38] or *in vivo* [39]. The diverse cytokine profiles of iNKT cells are known to be related to the nature of the antigen that stimulates them [38, 40]. For example, α-GalCer has been used to determine the functional significance of iNKT cell populations in the protection or prevention of autoimmune diseases such as type 1 diabetes and experimental autoimmune encephalomyelitis where protection of mice was associated with biased Th2 response [35, 40].

Stimulation of our iNKT cell lines with thyroglobulin or α-GalCer revealed only slightly different cytokine profiles between the two lines. After stimulation with either thyroglobulin or α-GalCer, both cell lines rapidly produced certain key cytokines such as IL-2, IL-4, IFN-γ, IL-10, and

Table 2: Surface phenotype of two lines of iNKT cells. Both lines expressed markers for T and NK cells.

Surface markers	1F1.1	2D11	CD4 (control)
T-cell markers			
TCR$\alpha\beta$	+	+	+
CD3	+	+	+
CD4	+	+	+
CD8	−	−	−
CD69	+	+	+
NK cell markers			
DX5	+	+	−
NK1.1	−	−	−
Ly6	+	+	−
APC markers			
CD1d	+	+	−
Mac1	−	−	−
CD80	−	−	−
CD86	−	−	−

TNF-α. Although the iNKT cell lines produced slightly different cytokine levels, both were capable of enhancing disease in genetically susceptible mice. Since iNKT cells were stimulated only with the thyroglobulin preparation, as presented by CD1d molecules, different epitopes of thyroglobulin could possibly be a ligand of iNKT cells. We thus suspect that in NOD·$H2^{h4}$ mice and possibly in susceptible humans, autoimmune thyroiditis is enhanced indirectly by the cytokine products of iNKT cells in response to thyroglobulin stimulation.

Although Th1-type $CD4^+$ T cells are considered to be the predominant contributors to the initiation and persistence of autoimmune thyroiditis [5, 41], our study using thyroglobulin-stimulated iNKT cell lines that express IL-2, IFN-γ, IL-10, and TNF-α implicate a role for iNKT cells in the enhancement of autoimmune thyroiditis. A significant increase in serum levels of IgG1 and IgG2b antibodies to thyroglobulin 14 days after adoptive transfer of iNKT cell lines further suggests a key role of iNKT cells in the enhancement of thyroidal autoimmunity. The precise mechanisms involved in the enhancement of autoimmune thyroiditis by these iNKT cell lines are still unclear. Work by other investigators has clearly demonstrated that NKT cells promote autoimmune disease under certain conditions [10, 11, 42, 43]. It has been suggested that the stage of disease in which NKT cells are introduced into the experimental system play a major role in the outcome of disease. For example, if given early, prior to the start of disease the result is protection, but if given after initiation of disease, the result is disease enhancement [44]. In our experimental model of iodine-induced thyroiditis in the NOD·$H2^{h4}$ mouse, transfer of the 1F1.1 iNKT cell line promoted disease only in iodine-primed mice, further suggesting that the role of these cells is enhancement rather than initiation of disease.

In summary, iNKT cell lines were derived from spleens of NOD·$H2^{h4}$ mice by repeated stimulation with a mouse thyroglobulin preparation. These cell lines have all the molecular and functional earmarks of iNK T cells. When these lines were transferred into iodine-primed NOD·$H2^{h4}$ recipients, thyroid autoimmunity was enhanced. In this model system of iodine-induced thyroiditis, these iNKT cells may be producing large quantities of Th1 cytokines, such as IFNg or TNFα, that dominate a more protective role of the Th2 cytokine response. These experiments lend further caution about NKT cell therapy for autoimmune diseases, as what may be protective in one could lead to disease enhancement in another.

Abbreviations

APC: Antigen-presenting cell
BM: Bone marrow
Ci: Curie
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
GM-CSF: Granulocyte/macrophage colony-stimulating factor
h: Hour
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
i.p.: Intraperitoneal(ly)
i.v.: Intravenous(ly)
mAb: Monoclonal antibody
NK cell: Natural killer cell
OD: Optical density
OVA: Ovalbumin
PBS: Phosphate-buffered saline
RT: Reverse transcription
PCR: Polymerase chain reaction
TCR: T-cell receptor for antigen.

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