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SHORT COMMUNICATION

MHC class II-expressing thymocytes suppress invariant NKT cell development

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Natural killer T (NKT) cells are positively selected on cortical thymocytes expressing the non-classical major histocompatibility complex (MHC) class I CD1d molecules. However, it is less clear how NKT cells are negatively selected in the thymus. In this study, we investigated the role of MHC class II expression in NKT cell development. Transgenic mice expressing MHC class II on thymocytes and peripheral T cells had a marked reduction in invariant NKT (iNKT) cells. Reduced numbers of iNKT cells correlated with the absence of *in vivo* production of cytokines in response to the iNKT cell agonist α-galactosylceramide. Using mixed bone marrow chimeras, we found that MHC class II-expressing thymocytes suppressed the development of iNKT cells *in trans* in a CD4-dependent manner. Our observations have significant implications for human iNKT cell development as human thymocytes express MHC class II, which can lead to an inefficient selection of iNKT cells.
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Natural killer T (NKT) cells are a specialized subset of T cells that coexpress NK cell markers, such as NK1.1. They originate in the thymus and are positively selected by CD1d on cortical CD4 and CD8 double-positive (DP) thymocytes.[1–3] There are at least two sub-populations of NKT cells based on their T-cell receptor (TCR) diversity.[1] The major subset is called invariant NKT (iNKT) cells because their TCR repertoire is highly limited, with the majority of them carrying the invariant Vα14–Jα18 α-chain paired mostly with one of the three Vβ chains (Vβ8.2, Vβ7 and Vβ2) in mice. The other subset has a diverse TCR repertoire and represents the non-invariant NKT cells.

Invariant NKT cells can be negatively selected when iNKT TCR receives too strong signal.[2–4] The addition of iNKT cell agonist α-galactosylceramide (α-GalCer) to fetal thymic organ culture leads to iNKT cell deletion,[5,6] and the transgenic expression of CD8α and CD8β chains in T cells also results in a reduction of iNKT cells.[7] Transgenic mice overexpressing the NK-activating receptor LY49D have reduced iNKT cell development.[8] Furthermore, the overexpression of CD1d in dendritic cells decreased the number of iNKT cells.[6,9]

Unlike murine NKT cells, the number of Vα24$^+$ iNKT cells in humans is highly variable among individuals and they represent only a small portion of total NKT cells, whereas non-invariant NKT cells are the major population.[1,10–14] Human thymocytes can express not only CD1 but also major histocompatibility complex (MHC) class II.[15] However, it is not known whether MHC class II on thymocytes plays a role during NKT cell development because murine thymocytes do not express MHC class II. In this study, we showed that MHC class II-expressing thymocytes suppress the development of iNKT cells partly due to the interaction of MHC class II with CD4.

RESULTS AND DISCUSSION

To study the effect of MHC class II expression on NKT cell development, we utilized mice that express MHC class II in thymocytes and peripheral T cells by introducing the MHC class II transactivator (CIITA) gene as a transgene (CIITA^{Tg}).[16] CIITA^{Tg} mice had reduced thymic NK1.1$^+$TCRβ$^+$ NKT cells but not in the periphery (Figure 1a). We next examined iNKT cells using CD1d tetramers loaded with α-GalCer (hereafter referred to as tetramers) that specifically identify iNKT cells. As shown in Figures 1b and c, CIITA^{Tg} mice exhibited a marked reduction in iNKT cells both in the thymus and the peripheral tissue, indicating that CIITA expression in T cells led to a selective failure of iNKT development and a corresponding expansion of non-invariant NKT cells. The reduction in iNKT cell number was MHC haplotype independent (Figure 1d). However, unlike *CD1d*$^{-/-}$ mice that had no iNKT cells, residual iNKT cells were present in CIITA^{Tg} mice (Figure 1b). These residual iNKT cells in the CIITA^{Tg} thymus seemed to have undergone proper developmental stages and become

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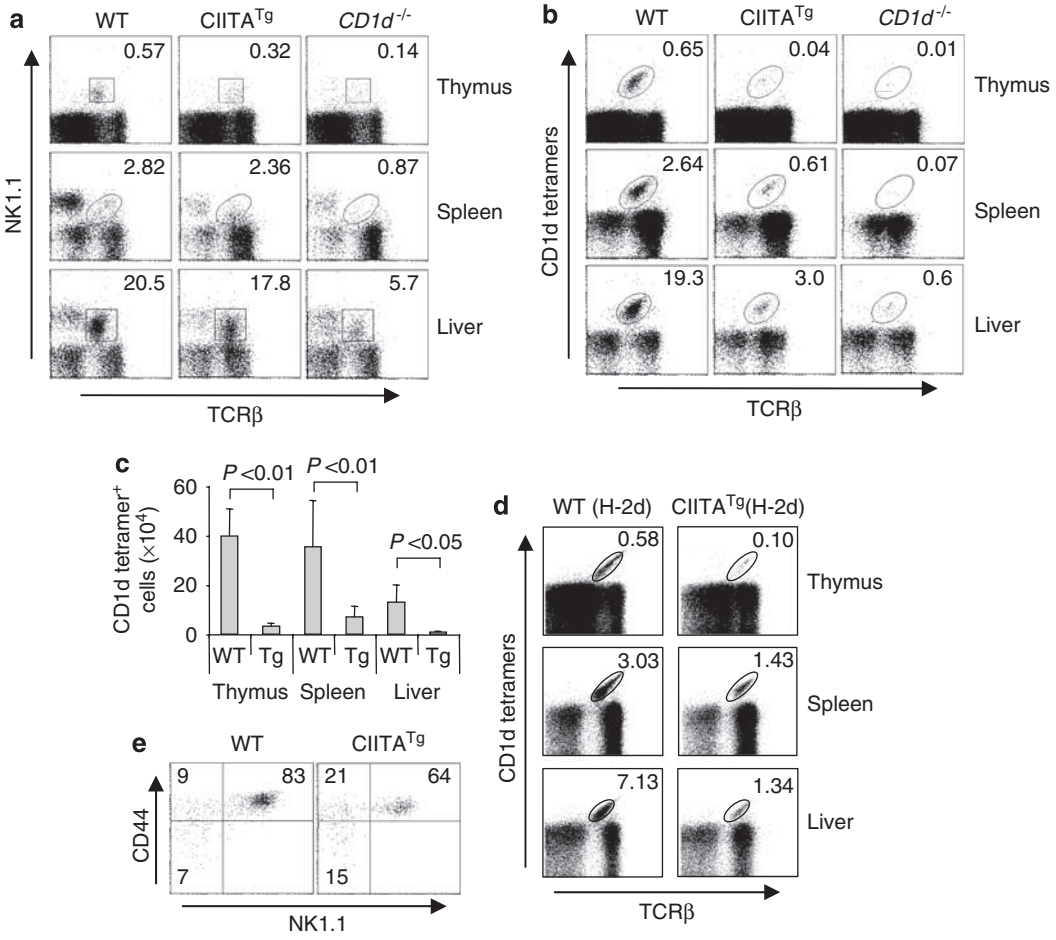

Figure 1 Reduction of iNKT cells in CIITA^{Tg} mice. Expression of TCRβ and NK1.1 markers (**a**) or TCRβ and tetramer (**b**) on indicated cell types from WT (CIITA^{Tg} littermates), CIITA^{Tg} and *CD1d*$^{-/-}$ mice. The number in the dot plots represents the percentage of NK1.1^{+}TCRβ^{+} NKT cells (**a**) or iNKT cells (**b**). Mice used were 10–11 weeks old and similar results were obtained from at least four mice per group. (**c**) Absolute numbers of iNKT cells. Values are mean ± s.d. of iNKT cells present in the thymi (n=10), spleens (n=6) and livers (n=4). Mice were 2–3 months old. (**d**) Representative iNKT cell staining of three pairs of WT (CIITA^{Tg} littermates) and CIITA^{Tg} on the H-2^{d} background. (**e**) Analysis of NK1.1 and CD44 expression on thymic iNKT cells. Tetramer^{+}HSA^{lo} cells were gated and percentages of cells in each quadrant are shown. Results are representative of four pairs of WT and CIITA^{Tg} littermates. Mice were 10 weeks old. iNKT cells, invariant natural killer T cells; TCR, T-cell receptor; WT, wild type.

mature iNKT cells as defined by CD44 and NK1.1 expression, albeit a slight increase and decrease in the percentages of immature and the most mature iNKT cells, respectively (Figure 1e). The decrease in iNKT cell number in the periphery resulted in very little circulating interleukin-4 or interferon-γ upon *in vivo* activation (Supplementary Figure 1a). The CD1d levels on CIITA^{Tg} DP thymocytes (Supplementary Figure 1b) were comparable with wild-type (WT) cells.

As CIITA can turn on the expression of multiple genes involved in MHC class II antigen presentation, such as the invariant chain (Ii),[17] we wanted to determine whether the expression of MHC class II or a gene product regulated by CIITA is responsible for the observed inefficient iNKT cell development. To do this, we generated CIITA^{Tg} mice lacking MHC class II by crossing them with MHC class II-deficient *Abb*$^{-/-}$ mice (CIITA^{Tg}/*Abb*$^{-/-}$). In these mice, developing thymocytes express the CIITA transgene but not MHC class II. The absence of MHC class II in CIITA^{Tg}/*Abb*$^{-/-}$ mice significantly improved the generation of iNKT cells (Figures 2a and b), suggesting that MHC class II participates in the development of iNKT cells. The incomplete restoration implies the possible involvement of other CIITA-regulated components in iNKT cell development. One putative candidate is Ii, which is known to associate with CD1d in endosomes and enhance CD1d antigen presentation of dendritic cells and B cells to iNKT cells.[18] Further study is required to determine whether Ii plays a suppressive role in iNKT cell development.

To test whether MHC class II-expressing thymocytes fail to develop into iNKT cells in a cell autonomous manner, we constructed mixed bone marrow (BM) chimeric mice. Different CD45 markers were used to distinguish cells derived from the two different BM sources and the host. In WT+WT→B6.SJL mice, each WT BM population generated a sizable iNKT cell population (Figure 2c, left group; Supplementary Figure 2). However, in the presence of CIITA^{Tg} BM, WT BM cells failed to generate a comparable proportion of iNKT cells, implying that MHC class II-expressing thymocytes suppress neighboring thymocytes to progress to iNKT cells *in trans* (Figure 2c, right group; Supplementary Figure 2).

To further substantiate the role of MHC class II in iNKT cell development, we cotransferred WT BM cells together with CIITA^{Tg}/*Abb*$^{-/-}$ BM to B6.SJL recipients. If MHC class II expression

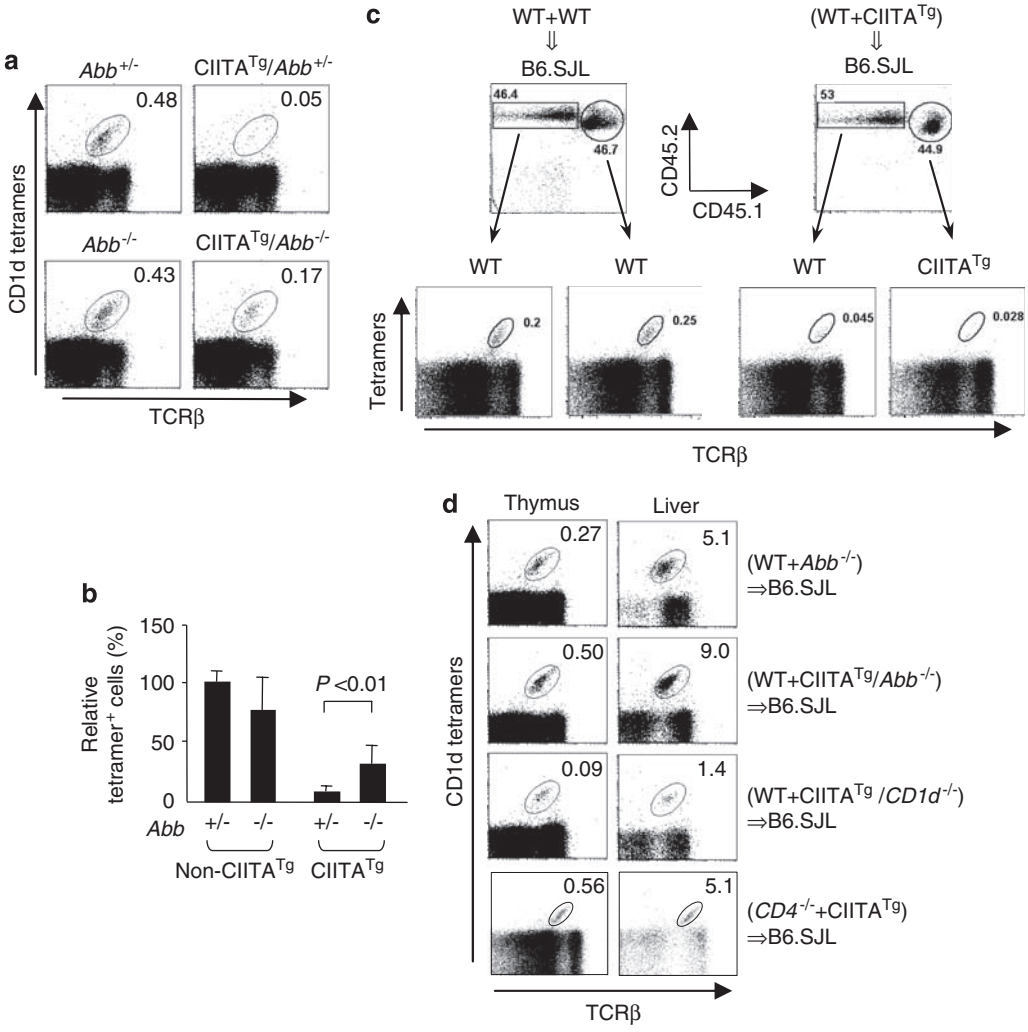

Figure 2 MHC class II inhibits iNKT cell development. (**a**) MHC class II expression in thymocytes is necessary for the inhibition of iNKT cell generation. Total thymocytes from the indicated mice were analyzed for TCRβ and tetramer reactivity. WT and CIITA^{Tg} mice on the *Abb*$^{+/-}$ and *Abb*$^{-/-}$ background were littermates. Results are representative of at least four experiments. (**b**) Relative percentages of thymic iNKT cells in at least eight mice of *Abb*$^{+/-}$, CIITA^{Tg}/*Abb*$^{+/-}$, CIITA^{Tg} /*Abb*$^{-/-}$ or *Abb*$^{-/-}$. The value of the percentage of iNKT cells in WT mice was set at 100%. Mice were 2–3 months old. (**c**) Suppression of iNKT cell development by MHC class II-expressing thymoyctes *in trans*. BM cells from WT (CD45.2) and WT (CIITA^{Tg} littermates; CD45.1/CD45.2) or WT (CD45.2) together with CIITA^{Tg} (CD45.1/CD45.2) were coinjected into B6.SJL (CD45.1) recipients. Shown at the top is the composition of total thymocytes in reconstituted mice. TCRβ versus tetramer profiles of thymocytes are shown at the bottom. Chimeras were analyzed 10–12 weeks post-BM transplantation. (**d**) MHC class II and CD4 expression is required for the disappearance of iNKT cells in mixed BM chimeric mice as indicated. BMs were prepared from WT (CD45.1/2), *Abb*$^{-/-}$ (CD45.2), CIITA^{Tg}/*Abb*$^{-/-}$ (CD45.2), CIITA^{Tg}/*CD1d*$^{-/-}$ (CD45.2), CIITA^{Tg} (CD45.1/2) and *CD4*$^{-/-}$ (CD45.2) mice and transferred to B6.SJL mice. The number in the dot plots represents the percentage of iNKT cells developed from WT BM cells except the bottom group that shows *CD4*$^{-/-}$ iNKT cells. Chimeras were analyzed 2–3 months post-BM transplantation. BM, bone marrow; iNKT, invariant natural killer T cells; MHC, major histocompatibility complex; TCR, T-cell receptor; WT, wild type.

itself is responsible for deleting thymocytes, iNKT cell development should be restored in WT+CIITA^{Tg}/*Abb*$^{-/-}$ →B6.SJL mice. Indeed, iNKT cells originated from WT BM were present at a normal level (compare the second row in Figure 2d with the right group in Figure 2c; Supplementary Figure 2). In the control WT+*Abb*$^{-/-}$→ B6.SJL chimeras, WT iNKT cells were also generated (Figure 2d, top row; Supplementary Figure 2). Earlier, overexpressing CD1d on DC that also express MHC class II led to a negative selection of iNKT cells.[6,9] Therefore, we asked whether coexpression of CD1d together with MHC class II on the same cells is necessary to delete iNKT cells. iNKT cells derived from WT BM were dramatically decreased in the presence of CIITA^{Tg}/*CD1d*$^{-/-}$ thymocytes, indicating the unlikely role of CD1d in the deletion of iNKT cells (Figure 2d, third row; Supplementary Figure 2). Finally, MHC class II on the thymocyte surface might function as a coreceptor for CD4 molecules, enhancing the avidity of TCR on NKT progenitors that are being selected. If so, the strength of signaling could reach beyond the threshold for the survival of cells. Indeed, in the *CD4*$^{-/-}$+CIITA^{Tg}→B6.SJL chimeras, CD4$^{-/-}$ thymocytes were developed to iNKT cells normally in the presence of MHC class II-expressing thymocytes (Figure 2d, bottom row; Supplementary Figure 2), suggesting that the interaction of MHC class II with CD4 is at least partly responsible for the disappearance of iNKT cells. Thus, in addition to its role in iNKT cell activation,[19,20] CD4 seems to participate in the regulation of iNKT cell development.

As iNKT cells are mainly composed of $CD4^+$ and $CD4^-CD8^-$ double-negative sub-populations, it would be interesting to test whether the interaction between MHC class II and CD4 would preferentially affect the development of $CD4^+$ iNKT subset.

Taken all together, we have demonstrated, for the first time, that MHC class II plays an important role during iNKT cell development. Similar to $CIITA^{Tg}$ mice, humans have much fewer iNKT cells than non-invariant NKT cells compared with mice. Perhaps, this is related to the fact that human fetal and neonatal thymocytes express MHC class II, which likely plays an inhibitory role in iNKT cell generation. The final tuning of the strength of the signal delivered by CD1d and/or MHC class II may be critical for survival or death of developing thymocytes to progress toward iNKT cells.

METHODS

Mice

Mice carrying the human type III CIITA transgene ($CIITA^{Tg}$), $CIITA^{Tg}$ mice on the MHC class II-deficient $Abb^{-/-}$ background ($CIITA^{Tg}/Abb^{-/-}$) and $CD1d^{-/-}$ on the C57BL/6 background were described earlier.[15,16,21] $CIITA^{Tg}$ and $CD1d^{-/-}$ mice were backcrossed with C57BL/6 (B6) mice at least six to eight times, respectively. $CIITA^{Tg}$ mice on the Balb/c background were backcrossed six times. $CD4^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and C57BL/6.SJL (B6.SJL) mice from Taconic (Germantown, NY, USA). Non-$CIITA^{Tg}$ littermates from heterozygous $CIITA^{Tg}$ breeding were used as WT controls. $CIITA^{Tg}$ and WT mice were also bred to carry CD45.1 and CD45.2 congenic markers. All mice were housed in the animal facility at the Indiana University School of Medicine or the University of Michigan Medical School under specific pathogen-free conditions and used at 6–12 weeks of age. All animal experiments were performed under protocols approved by the institutions.

Mixed bone marrow chimeras

Preparation of mature lymphocyte-depleted BM cells and BM transfers were performed as described earlier.[15] To generate mixed BM chimeras, BM cells from two different types of donor mice were mixed at a ratio of 1:1 and each lethally irradiated recipient mouse (950 rad) was infused with $2–5\times10^5$ cells through tail vein injection.

Flow cytometry

All antibodies used for flow cytometry were purchased from BD Biosciences (San Diego, CA, USA). Cells were pre-incubated with the anti-FcγR mAb 2.4G2 to block nonspecific antibody binding before staining with the following fluorescein isothiocyanate-, phycoerythrin-, PerCP-, CyChrome-, allophycocyanin- or biotin-conjugated antibodies: TCRβ (H57), CD4 (L3T4), B220 (RA3-6B2), NK1.1 (PK136), CD1d (1B1), CD45.1 (A20) and CD45.2 (104). Fluorochrome-conjugated streptavidin was used to visualize staining by biotinylated primary antibodies. Allophycocyanin-conjugated CD1d-α-GalCer tetramers were generously provided by Dr Albert Bendalac from the University of Chicago and the NIH Tetramer Facility. Events were acquired on a FACSCalibur or FACSCanto (BD Biosciences) flow cytometer and the data were analyzed using the CELLQuest Pro or FlowJo software.

In vivo cytokine production assay

Mice were injected i.v. with 2 μg α-GalCer[22] or vehicle in 100 μl of phosphate-buffered saline. After 2 h, mice were killed and serum interleukin-4 and interferon-γ were detected using enzyme-linked immunosorbent assay kits (BD Biosciences).

Statistical analysis

The non-parametric Wilcoxon rank sum test was performed to calculate statistical significance. *P*-values less than 0.05 were considered statistically significant.

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Supplementary Information accompanies the paper on Immunology and Cell Biology website (http://www.nature.com/icb)