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Soluble CD4 suppresses T-dependent IgG2a antibody response of CD4 loosing mice by inhibiting IFNγ production

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To analyze the role of soluble CD4 (sCD4) in antibody (Ab) responses in CD4 loosing (CD4L) mice, experiments have been done in comparing CD4L mice with CD4 knockout (CD4KO) mice on the same C57BL/6 background. The CD4L mice have a defect in CD4 expression where CD4 mRNA is alternatively spliced so that a transmembrane portion is deleted and sCD4 are secreted without expression of membrane-bound CD4. Significantly reduced immunoglobulin (Ig) G2a isotype Ab response against a T-dependent antigen (Ag), trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH), was found in CD4L mice as compared with those of CD4KO mice. Gamma interferon (IFNγ) production of KLH-stimulated lymph nodes cells was significantly reduced in CD4L mice as compared with those in CD4KO mice. The positive proportion of cells expressing CD40 ligand from CD4KO mice or CD4L mice was much lower than those from wild type mice. There was no difference between CD4KO and CD4L mice in T-cells expressing CD40 ligand after stimulation. Taken together, results in this study indicate that sCD4 suppresses IgG2a Ab responses of CD4L mice by inhibiting IFNγ production. The mutant mice could provide a good model to analyze the mechanism of IFNγ involvement in the Ab response against T-dependent Ag.

Key words: CD4 loosing mice, IFNγ, IgG2a antibody response, soluble CD4

CD4 molecule is a 55 kDa membrane glycoprotein which contains an extracellular region, a transmembrane portion and a cytoplasmic tail [1]. The extracellular region with amino acid sequences similar to immunoglobulin (Ig) superfamily comprises four domains. D1 and D2 domains are implicated in the binding of non-polymorphic region of major histocompatibility complex (MHC) class II molecules, and D1 and D4 domains are involved in oligomerization and dimerization of CD4 molecules [2,3]. CD4 molecules contribute to the overall stability and increase the association avidity of T-cell receptor (TCR)-MHC class II interaction [2]. CD4 dimerization and oligomerization are involved in the TCR-based signal transduction necessary for effective second messenger accumulation and induction of effector function [3]. The cytoplasmic tail is associated with tyrosine-protein kinase $p56^{lck}$ and CD4 can transmit signal by induction of $p56^{lck}$ kinase activity during antigen (Ag)-mediated peripheral T-cell activation [4,5]. CD4 molecules play key roles in the activation of peripheral T-cells [4-6]. Some antiCD4 monoclonal antibody (mAb) blocked cellular activation of T helper (TH) clones by suppression of interleukin (IL)-2 production [7]. Peripheral T-cells in CD4 knockout (CD4KO) mice generated by homologous recombination were shown to be impaired markedly in most of their immune functions such as mixed lymphocyte reaction and helper activity for antibody (Ab) production [8].

Soluble CD4 (sCD4) has been reported to be secreted into serum in patients with infections such as acquired immunodeficiency syndrome (AIDS) [9], infectious mononucleosis [10] and leishmaniasis [11], or with some autoimmune diseases such as rheumatoid arthritis [12] and Sjögren syndrome [13]. Mechanisms as to how sCD4 molecules are secreted from cells in patients with these diseases remain unclear. Release of lymphoid cell surface molecules was reported to occur by several different mechanisms. Activated lymphoid cells could shed CD25 into the circulation as a result of the stimulation of proteolytic enzymes [14]. Alternative splicing of the messenger RNA (mRNA) to exclude an exon encoding a transmembrane domain of CD8 molecule was reported to be responsible for the release of sCD8 [15]. Although several T-cell surface molecules including CD4, CD8 and CD25 secreted into circulation are claimed to be used for monitoring activity of immune mediated diseases, function of these soluble

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molecules remains to be studied.

A mutant mouse of the C57BR/cdJ background found in Japan [16] has a novel defect in CD4 expression where CD4 mRNA is alternatively spliced so that a transmembrane portion is deleted and sCD4 molecules are secreted without expression of membrane-bound CD4. These mutant mice were backcrossed onto C57BL/6 mice to the fifth generation and were named CD4 loosing (CD4L) mice [17]. In these mice, delayed-type hypersensitivity (DTH) response to methylated bovine serum albumin was severely impaired; however, CD4KO mice showed a response comparable to wild type mice [17]. In order to analyze in detail the effect of sCD4 on Ab production in the mutant mice, experiments have been carried out by comparing the CD4L mice with CD4KO mice. When Ab production against T-dependent Ag was examined, an apparent difference was found between these two strains of mice. The CD4L mice showed significantly reduced IgG2a Ab titers against trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) as compared with those in CD4KO mice.

In the present studies, results indicate that sCD4 molecules suppress IgG2a isotype Ab response against T-dependent Ag in mutant mice by blocking gamma interferon (IFNγ) production. The mutant mice could provide a good model to analyze the mechanism of IFNγ involvement in the T-dependent Ab response.

Materials and Methods

Mice

A mutant substrain of C57BR/cdJ mice with sCD4 but without membrane-bound CD4 has been backcrossed onto C57BL/6 mice to the fifth generation [17]. These mice are CD4L mice. CD4KO mice on C57BL/6 background [8] were a generous gift from Dr. T. Uchiyama, Tokyo Women Medical College, Tokyo, Japan, with permission from the original developer Dr. T.W. Mak, University of Toronto, Ontario, Canada. Wild type C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). In these experiments, five mice per group were used at 7 to 12 weeks of age.

Reagents

Anti-CD3ε (145-2C11, hamster IgG) was purified from ascites on a protein A column. Purified anti-CD4 (H129-19, rat IgG2a), fluorescein isothiocyanate (FITC)-labeled anti-CD4 (RM4-5, rat IgG2a) and biotin-labeled anti-CD4 (RM4-4, rat IgG2b) were purchased from PharMingen (San Diego, CA, USA). FITC-anti-B220 (RA3-6B2, rat IgG2a), phycoerythrin (PE)-anti-CD8 (53.6.7, rat IgG2a), FITC-anti-CD3ε (145-2C11), biotinylated anti-CD40 (3/23, rat IgG2a) and biotinylated anti-CD40 ligand (MR1, hamster IgG) were also purchased from PharMingen. Abs for capture and detection in IFNγ assay (R4-6A2, rat IgG1 and XMG1.2) were purchased from PharMingen. Biotinylated anti-mouse IgM and IgG1, IgG2a, IgG2b and IgG3 subclasses Abs were all purchased from Zymed (San Francisco, CA, USA). Magnetic particles coated with goat anti-rat IgG were obtained from Miltenyi Biotech (Germany). Anti-MHC class II (M5.114, rat IgG2b), anti-FCγR II/III (2.4.G2, rat IgG2b) and anti-HSA (M1.69, rat IgG2b) were all used in the form of culture supernatants during the experiment procedures. Horseradish peroxidase-labeled streptavidin was purchased from Zymed. PE-streptavidin was obtained from Gibco (Gaithersburg, MD, USA) and streptavidin-cy-chrome was purchased from PharMingen. Propidium iodide was obtained from Sigma. Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were purchased from Difco (Detroit, MI, USA). KLH was purchased from CalBiochem (La Jolla, CA, USA) and trinitrobenzene sulfonate (TNBS) was from Wako (Osaka, Japan). TNP-KLH was prepared by conjugation of TNBS to KLH.

Ab production assay

Mice were immunized intraperitoneally with a T-dependent Ag, TNP-KLH, 100 μg per mouse emulsified in FCA and boosted intraperitoneally 3 weeks later with the same amount of TNP-KLH in FIA. Mice were bled every 7 days and serum samples were analyzed for anti-TNP Ab by enzyme-linked immunosorbent assay (ELISA) in the plate coated with dinitrophenyl body surface area (DNP-BSA). The TNP-specific IgM Ab and subclasses of the TNP-specific IgG1, IgG2a, IgG2b and IgG3 Ab were detected by an incubation with biotinylated anti-mouse Ab (Zymed), followed by the addition of horseradish peroxidase-labeled streptavidin (Zymed), and finally the wells were developed with substrate peroxidase [18].

FACS analysis

One million cells in single cell suspension from lymph node or spleen or 3×10^5 $CD4^+$ T-cells or double negative (DN) T-cells were analyzed on a FACScan using Lysis II software (Becton Dickinson, Mountain View, CA) for data analysis. These cells were first incubated with anti-FCγR II/III (2.4.G2) to block binding of Ab to Fc receptor before analysis and then incubated with labeled mAb at 4 °C for 30 min. Dead cells positively stained with propidium iodide (Sigma)

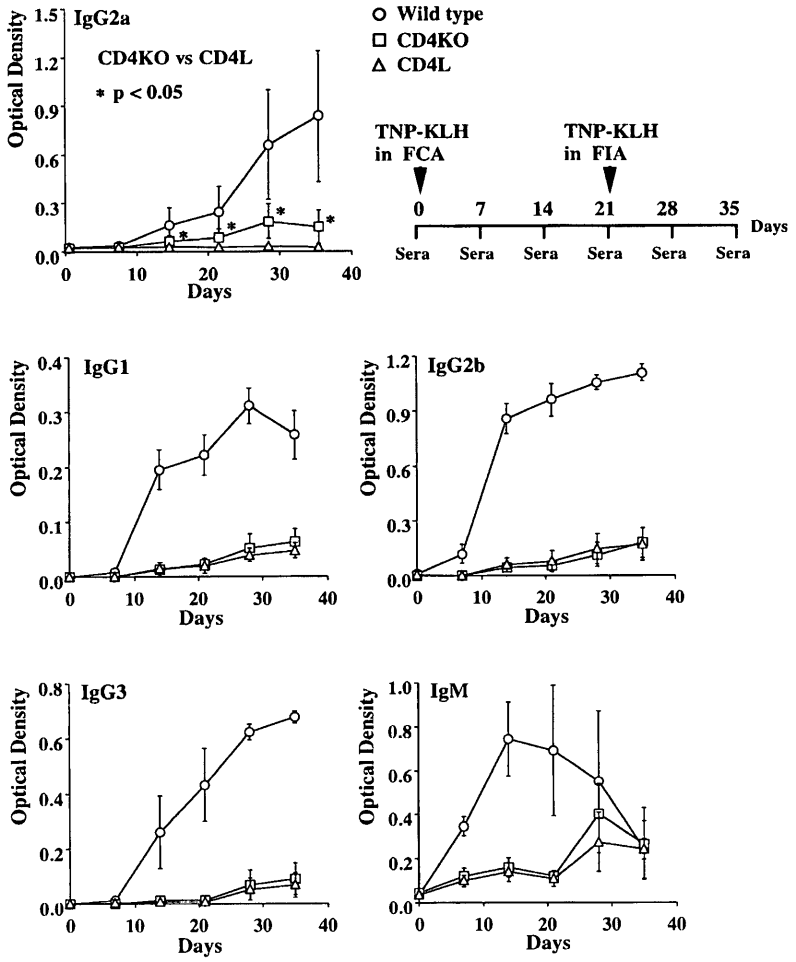

Fig. 1. Ab production of CD4L, CD4KO and wild type mice. Mice were immunized intraperitoneally with a T-dependent Ag, TPN-KLH, 100 μg/mouse emulsified with FCA and boosted intraperitoneally 3 week later with the same amount of Ag in FIA. Mice were bled every week and serum samples were analyzed for anti-TNP by ELSIA. The data are shown as mean ±SD (*$p < 0.05$).

were gated out. For the three-color analysis, cells stained with biotinylated Ab were incubated with streptavidin-cy-chrome at 4 °C for 20 min.

Assay for IFNγ production

Popliteal lymph node cells from mice primed for 7 days by a footpad injection with 50 μg KLH emulsified with FCA were stimulated *in vitro* at 6 x 10^6 cells/culture with 300 μg/mL KLH in a well of flat bottom 24 well plate, and culture supernatants were assayed for IFNγ in sandwich ELISA using different clones of mAb for capture (R4-6A2) and detection (XMG1.2). The assay was performed according to the manufacturer's instruction (PharMingen).

Induction of CD40 ligand expression on DN T-cells

T-cells were enriched by passing the spleen cells through a nylon wool column. The cells passed through the column were incubated with a mixture of anti-CD8 (53.6.7), anti-MHC class II (M5.114), anti-FCγR II/III (2.4.G2) and anti-HSA (M1.69), and then treated with magnetic particles coated with anti-rat IgG (Miltenyi Biotech) and depleted of the cells with these markers to enrich the $CD4^+$ T-cells or DN T-cells. $CD4^+$ T-cells from wild type mice and DN T-cells from CD4KO or CD4L mice were stimulated by plate-coated anti-CD3ε (145-2C11) 1 μg/well in a well of flat bottom 48 well plate. The biotinylated anti-CD40 ligand (MR1) or biotinylated hamster IgG were simultaneously added into culture media with concentration of 1 μg/mL to prevent down-regulation of CD40 ligand expression, when these cells were stimulated [19]. After stimulation for 14 h, cells were doubly stained with anti-Thy 1.2 (30-H12) and anti-CD40 ligand (MR1) and were

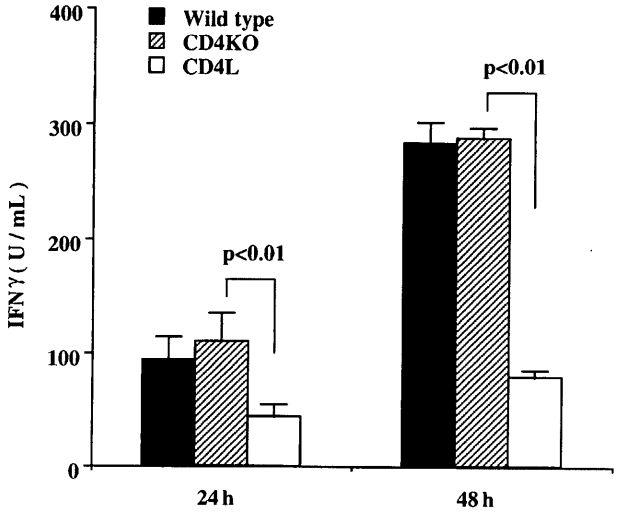

Fig. 2. IFNγ production of CD4L, CD4KO and wild type mice. Popliteal lymph node cells from mice primed for 7 days by a footpad injection with 50 μg KLH emulsified with FCA were stimulated *in vitro* at 6 x 10^6 cells/culture with 300 μg KLH in a well of flat bottom 24 well plate, and culture supernatants were assayed for IFNγ by ELISA. The data are shown as mean ±SD.

analyzed on a FACScan. In addition, the expression of CD40 on B-cells was examined by doubly staining the spleen cells with anti-B220 (RA3-6B2) and anti-CD40 (3/23).

Statistical analysis

Results are expressed as mean ±SD. The Student's *t*-test was used to calculate probability (p) values. A p-value less than 0.05 was considered significant. The statistical analyses were undertaken using the Microsoft Excel program.

Results

Reduced T-dependent IgG2a Ab response in CD4L mice

The Ab production against a T-dependent Ag, TNP-KLH, in wild type, CD4KO and CD4L mice were examined. Both CD4KO and CD4L mice produced significantly lower Ab titers of IgM class and all IgG subclasses 2 weeks after the primary immunization, as compared with those in wild type mice ($p < 0.01$) (Fig. 1). Except IgG2a isotype, there was no difference in Ab titers between CD4KO and CD4L mice ($p > 0.1$). The IgG2a isotype Ab titers were significantly higher in CD4KO mice than those in CD4L mice ($p < 0.05$).

IFNγ production of Ag-stimulated lymph node cells

IFNγ has been well documented to be involved in T-dependent Ab production [20]. In the present study, Ag-induced IFNγ production of lymph node cells from wild type, CD4KO and CD4L mice were analyzed. Mice were primed by hind footpad injection of KLH in FCA. Seven days later, popliteal lymph node cells were stimulated *in vitro* with KLH, and supernatants were assayed for IFNγ by ELISA. Lymph node cells from CD4L mice produced significantly lower amount of IFNγ ($p < 0.01$) as compared with that in CD4KO or wild type mice (Fig. 2). Lymph node cells from CD4KO mice produced similar amount of IFNγ in comparison to that from wild type mice ($p > 0.1$). To confirm the reduced production of IFNγ by lymphoid cells from CD4L mice, spleen cells from wild type, CD4KO and CD4L mice were stimulated with anti-CD3ε. The cells from CD4L mice produced significantly lower amounts of IFNγ in comparison to that from wild type or CD4KO mice (data not shown). These results indicate that IFNγ production is severely impaired in cells derived from CD4L mice in comparison to that by cells derived from CD4KO or wild type mice.

Expression of CD40 and CD40 ligand

Interaction between CD40 and CD40 ligand has been shown to be a critical step in Ig class switching against T-dependent Ag [21]. Therefore, the expression of CD40 and the induction of CD40 ligand were examined on wild type, CD4KO and CD4L mice. The intensities of CD40 fluorescence on B-cells from the spleen were similar among three groups of mice. The CD40 ligand was not expressed on CD8 T-cells after anti-CD3ε Ab stimulation (data not shown). The positive proportion of cells expressing CD40 ligand after anti-CD3ε Ab stimulation on DN T-cells from CD4KO mice (34.5%) or CD4L mice (34.2%) was lower than those on $CD4^+$ T-cells from wild type mice (70.6%); however, there was no difference in positive proportion between CD4KO and CD4L mice (Fig. 3).

Discussion

The CD4KO mice mounted reduced Ab responses against T-dependent Ag as compared with those in control mice [22,23]. In CD4L mice, Ab responses to a T-dependent Ag, TNP-KLH, were quite similar to those in CD4KO mice except IgG2a Ab response. A low but significant IgG2a Ab response was observed in CD4KO mice. However, IgG2a Ab response was severely impaired in CD4L mice. IFNγ was reported to selectively induce IgG2a production by resting B-cells stimulated with lipopolysaccharide (LPS) *in vitro* [24], and IgG2a production by Ag-specific B-cells were known to be mainly helped by TH_1 cells [25]. Some virus-specific IgG2a Ab production was shown to be

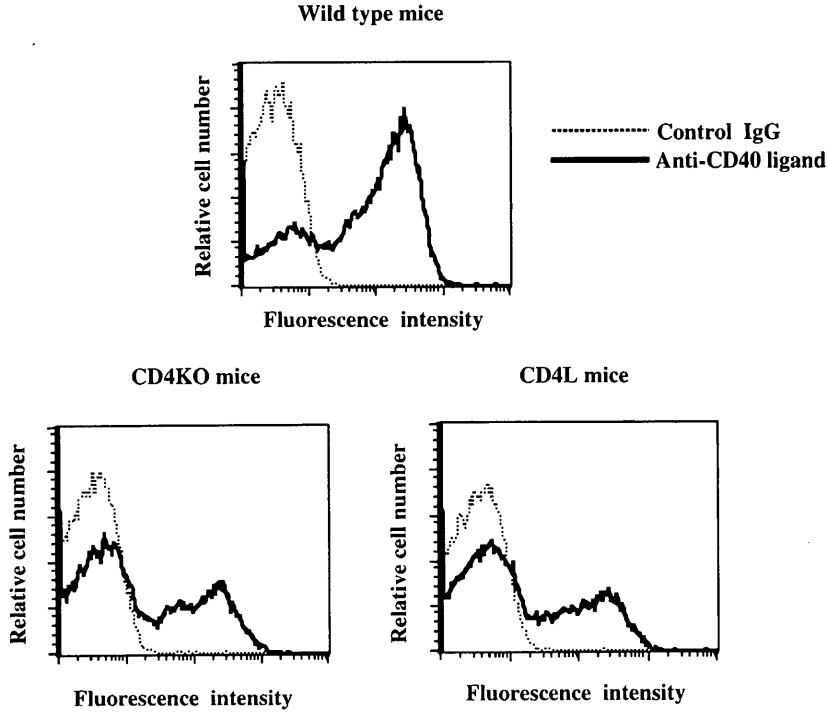

Fig. 3. CD40 ligand expression of DN T-cells from CD4L, CD4KO and wild type mice. Purified $CD4^+$ T-cells from wild type mice and DN T-cells from CD4KO or CD4L mice were stimulated for 14 h with anti-CD3ε coated in 48 flat bottom well plates (1 μg/well). To prevent down-regulation of CD40 ligand expression, 1 μg/mL biotinylated anti-CD40 ligand or hamster IgG was also added to each well. CD40 ligand expression was analyzed on Thy 1.2 positive cells on a FACScan.

decreased in IFNγ deficient mice [26]. Moreover, protein Ag-specific IgG2a Ab production could not be detected in the same strain of mice [27]. The IgG isotypes in Ab production may depend on the ratio of TH_1 and TH_2 activation, with a TH_1 bias giving preferentially IgG2a and a TH_2 preference resulting in more IgG1 [20].

Cognate interaction between T- and B-cells is indispensable for the production of Ab against T-dependent Ag [25]. The CD40 ligand KO mice, though with a weak IgM Ab response, failed to generate T-dependent Ag specific IgG isotypes Ab responses [18]. *In vivo* administration of anti-CD40 ligand significantly reduced both primary and secondary humoral immune responses to protein Ag [28]. No difference was found in the expression of CD40 and CD40 ligand between CD4KO and CD4L mice. These results indicate that CD40 and CD40 ligand interaction does not contribute to the difference in IgG2a isotype Ab production between CD4KO and CD4L mice. Other mechanisms such as reduced Ag-induced IFNγ production might be involved in the impaired IgG2a Ab production in CD4L mice.

Several investigators indicated that MHC class II molecule binding ligands could influence the proliferation of B-cells and inhibit the *in vitro* cytokine production by lymphocytes [29,30]. The MHC class II molecules might not act simply as a recognition structure of Ag presentation but also be able to perform a signal transduction in the regulation of Ag-presenting cells activity. sCD4 in CD4L mice was confirmed to bind MHC class II molecules (unpublished data). There is a possibility that binding of sCD4 to MHC class II molecules inhibits the activation of IFNγ-producing cells such as $CD4^-CD8^-$ DN T-cells, $NK1.1^+$ T-cells and $CD8^+$ T-cells.

Results in the present experiments indicate that sCD4 suppresses IgG2a Ab response against T-dependent Ag in CD4L mice by blocking IFNγ production. It is possible that, by introducing appropriated concentration of sCD4 into wild type mice, sCD4 could compete with surface CD4 for binding to MHC class II molecules. Through such binding, MHC class II-restricted $CD4^+$ T-cells could be inhibited in their ability in producing IFNγ and IgG2a Ab response might be suppressed in wild type mice. In conclusion, by using the CD4L mice model in analyzing the Ab response, the results of the present study could gain

some insight into the human diseases associated with increased sCD4 levels.

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