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Elevated IL-6 expression in CD4 T cells via PKC θ and NF- κ B induces Th2 cytokine production

M. Hanief Sofi^a, Wei Li^b, Mark H. Kaplan^c, and Cheong-Hee Chang^{a,*}

^a Department of Microbiology and Immunology, The University of Michigan Medical School, Ann Arbor, MI 48109, United States

^b Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, United States

^c Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN 46202, United States

Abstract

T helper (Th) cell differentiation is a key event to mount an appropriate immune response. Th1 and Th2 cells produce their signature cytokines IFN- γ and IL-4, respectively. However, as we have reported, CD4 T cells selected by MHC class II-expressing thymocytes (T-CD4) produce both Th1 and Th2 type cytokines under Th1 differentiation conditions. Furthermore, the expression of Th2 cytokines in these cells is Stat6 independent. In the current study, we investigated the molecular mechanisms by which CD4 T cells produce Th2 cytokines under the Th1 differentiation condition. We observed that IL-6 is highly expressed in T-CD4 T cells, which is at least partly responsible for Th2 cytokine production by Th1 differentiated cells. The enhanced expression of IL-6 is downstream of constitutive phosphorylation of PKC θ and high NF- κ B activity. Neutralizing IL-6, blocking PKC θ phosphorylation, or inhibiting NF- κ B translocation diminished Th2 cytokine expression in Th1 cultures. Therefore, our study revealed that autocrine IL-6 production can induce Th2 cytokine production, and that PKC θ and NF- κ B are essential components in the induction of IL-6-mediated Th2 development.

Keywords

Rodents; Cytokines; T cells; Signal transduction

1. Introduction

Host immune response against infection by different microbes as well as the prevention of autoimmune and allergic diseases requires tailored immune strategies. To accommodate this, depending on the nature of signals encountered during initial stimulation, naïve CD4 T cells are differentiated towards T helper (Th) 1, Th2, and Th17 cells, distinguished primarily by the potential to express a different set of cytokine genes (Harrington et al., 2005). Th1 and Th2 cells producing the hallmark cytokine IFN- γ and IL-4, respectively, are primarily responsible for cellular and humoral immunity (Murphy and Reiner, 2002). The signature cytokine produced by Th17 is IL-17 that is considered as an inflammatory cytokine (Park et al., 2005).

* Corresponding author at: Department of Microbiology and Immunology, The University of Michigan Medical School, 5641 Medical Science Bldg II, Ann Arbor, MI 48109-0620, United States. Tel.: +1 734 647 7570; fax: +1 734 764 3562. heechang@umich.edu (C.-H. Chang).

Because of the significance of Th effector cells during an immune response, the molecular mechanisms regulating Th cell generation has been extensively studied over years.

Many factors, including the dose of antigen and the type of antigen presenting cells (APC), the co-stimulatory molecules, and cytokines present at the time of T cells activation, are known to influence Th differentiation of CD4 T cells (Constant and Bottomly, 1997). Among them, the most effective inducer of CD4 T cell differentiation is the local cytokine environment. It is clear that IL-12 and IL-4 directs differentiation of naïve CD4 T cells to Th1 and Th2 cells, respectively. For Th2 cell differentiation, the physically clustered Th2 cytokine genes *Il4*, *Il13*, and *Il5* are transcriptionally activated upon receiving IL-4-mediated signals. IL-4 receptor ligation induces Stat6 activation followed by GATA3 expression, which subsequently mediates chromatin remodeling of the IL-4 locus (Avni et al., 2002). Therefore, in the absence of either IL-4 or Stat6, both expression of Th2 cytokine genes and the mounting of Th2 immune responses are greatly diminished (Chen et al., 2003; Kaplan et al., 1999). Similarly, Stat4 and T-bet are key transcriptional activators of the *Ifng* gene to generate Th1 cells (Yu et al., 2007).

We and others have demonstrated that CD4 T cells can be selected by MHC class II-expressing thymocytes (Choi et al., 2005; Li et al., 2005) and these thymocyte-selected CD4 (T-CD4 hereon) exhibit the unusual effector function (Li et al., 2007). Unlike CD4 T cells that are developed by thymic epithelial cells (E-CD4 T cells hereon), T-CD4 T cells produce Th1 and Th2 effector cytokines shortly after activation *in vivo* and *in vitro* (Li et al., 2007). Strikingly, T-CD4 T cells can produce both Th1 and Th2 type cytokines under non-skewing, Th1, differentiation conditions (Li et al., 2007).

T-CD4 T cells deficient in IL-4 expression can produce IL-5 and IL-13, eliminating IL-4 as a required Th2 inducing cytokine in T-CD4 T cells (Patel et al., 2005). In addition, the IL-4 gene is expressed as T-CD4 T cells are generated in the thymus, which does not require Stat6 (Li et al., 2007). Together, the molecular mechanisms regulating Th2 cytokine gene expression in T-CD4 T cells seem to be distinct from that in E-CD4 T cells.

IL-6 is known to polarize naïve CD4 T cells into Th2 cells by up-regulating NFATc2, which in turn promotes Th2 differentiation by inducing the initial expression of IL-4 (Diehl et al., 2002). Similarly, CD4 T cells from IL-6^{-/-} mice failed to produce IL-4 upon stimulation unless exogenous IL-6 was provided (Rincon et al., 1997). In contrast, CD4 T cells produce IL-4 when they were activated with IL-6^{-/-} APC (Rincon et al., 1997). Therefore, CD4 T cells have the intrinsic capability to produce IL-4 by expressing their own IL-6 upon activation.

In the present study, we showed that IL-6 made by CD4 T cells is essential to activate IL-4 gene expression in E- and T-CD4 T cells when exogenous IL-4 is not available. This IL-6-mediated induction of IL-4 expression requires PKCθ and its downstream molecule NF-κB but not MAPK. Furthermore, T-CD4 T cells produced higher amounts of IL-6 and showed greater phosphorylation of PKCθ than E-CD4 T cells. Neutralizing IL-6 or blocking PKCθ significantly reduced Th2 cytokine expression by T-CD4 T cells suggesting that enhanced production of IL-6 in T-CD4 T cells is at least partly responsible for the constitutive expression of Th2 cytokines.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). CIITA transgenic mice were previously described (Patel et al., 2005). All mice were housed under specific pathogen free conditions at the Indiana University School of Medicine and the

University of Michigan Medical School in accordance with institutional guidelines. Mice were used at 6–12 weeks of age and all animal experiments were performed under protocols approved by the Animal Care and Use Committee in both institutions.

2.2. Reagents and antibodies

Rottlerin and SN50 were purchased from Cal Biochem (La Jolla, CA). U0126 and SP600125 were obtained from Promega (Madison, WI) and Sigma–Aldrich (St. Louis, MO), respectively. Anti-IL-6 antibody was purchased from eBioscience (San Diego, CA). Antibodies recognizing all MAPK and anti-p-PKC θ (Thre538) were from Cell Signaling Technology (Beverly, MA). Anti-ERK and PKC θ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Preparation and stimulation of CD4 T cells

Total peripheral CD4 cells were enriched from single cell suspensions from spleen and lymph nodes (auxiliary, brachial, inguinal, and mesenteric) with anti-mouse CD4 microbeads (Miltenyi Biotec, Auburn, CA). To obtain naïve CD4 T cells, enriched CD4 T cells were stained with anti-CD4, CD45RB, and CD44 antibodies, and sorted electronically for CD4⁺CD45RB^{hi}CD44^{lo}. The purity of naïve cells was typically >95%. To induce Th differentiation, total peripheral CD4 T cells or naïve CD4 T cells (1×10^6 ml⁻¹) were stimulated with 5 μ g/ml plate-bound anti-CD3 ϵ (145-2C11), 1 μ g/ml anti-CD28 (37.51) antibodies and 50 U of IL-2 (Roche, Indianapolis, IN) for 5–7 days. For Th1 differentiation, 3.5 ng/ml of IL-12 and 10 μ g/ml of anti-IL-4 (11B11) antibody were added. Th2 cultures were supplemented with 10 ng/ml of IL-4 and 10 μ g/ml of anti-IFN- γ (R4-6A2) antibody.

2.4. Cytokine assays

For the ELISA assays, differentiated cells were re-stimulated overnight with 5 μ g/ml of plate-bound anti-CD3 antibody at a cell density of 1×10^6 ml⁻¹. IL-4, IL-5, IL-6, IL-13, and IFN- γ in the supernatants were quantified by paired cytokine-specific antibodies that were purchased from BD Pharmingen (San Diego, CA) or eBioscience. Recombinant cytokines were used as standards. For intracellular cytokine staining, cells were stimulated with 50 ng/ml phorbol myristyl acetate and 1.5 μ M ionomycin (Calbiochem) for 5 h. Monensin (Sigma–Aldrich) at 3 μ M was added during the last 3 h of stimulation. Cells were then fixed in 2–4% paraformaldehyde, and permeabilized with 0.2% saponin (Sigma–Aldrich), followed by staining with anti-IL4 (11B11) and anti-IFN- γ (XMG1.2) antibody for flow cytometry.

2.5. PCR

Total RNA was extracted from CD4 T cells lysed in TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacture's recommendations, and reverse transcribed using the Superscript First-strand cDNA synthesis system (Invitrogen Life Technologies). The following primer pairs for RT-PCR were used. Forward: 5'-CATCGGCATTTTGAACGAGGTCA-3' and reverse: 5'-CTTATCGATGAATCCAGGCATCG-3' for IL4; forward: 5'-CATTGAAAGCCTAGAAAGTCAG-3' and reverse: 5'-CTCATGAATGCATCCTTTTTCG-3' for IFN- γ and forward: 5'-GCTGGTGAAAAGGACCTCT-3' and reverse: 5'-CACAGGACTAGAACACCTGC-3' for HPRT.

2.6. Western blot

Protocols for the preparation of total cell lysates and immunoblotting were previously described (Sisk et al., 2000). Briefly cells were lysed with 1 $\times$ SDS sample buffer. Protein was separated by 10% SDS-PAGE and electroblotted onto Hybond nitrocellulose membrane. The membrane

was then incubated with anti-phospho antibody and developed by using the Amersham ECL system, according to the manufacturer's instructions. As a loading control, the blot was stripped and re-probed with anti-total protein antibodies.

2.7. Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation analysis was performed according to the Upstate Biotechnology, Inc. (Waltham, MA) Chromatin Immunoprecipitation (ChIP) assay protocol. Briefly, 2×10^6 differentiated Th1 cells from WT control and CIITA^{Tg} Th1 cells were fixed in 1% formaldehyde for 10 min at room temperature, washed, lysed and sonicated with three pulses to generate chromatin fragments of approximately 500 bp in length. Anti-acetylated histone H3 (Upstate Biotechnology, Inc.) antibody was added (3 μ l per immunoprecipitation) to the diluted lysates and incubated overnight. No antibody control was used as a negative control. Protein A-Sepharose CL-4B beads (Amersham Biosciences, Piscataway, NJ) were then added for 1 h. After washes, the immunocomplex were eluted, the cross links were reversed and DNA was purified by phenol/chloroform extraction and suspended in 50 μ l of TE buffer. Semi-quantitative PCR was done on these samples. The primers used were: IL-4 intronic enhancer (IE) 5'-GGGTGTGAATAAAGCCATATTG-3' and 5'-CCCAGCGTTTACATGAGC-3' and CD3 ϵ 5'-CATTTCCAAGTGACGTGG-3' and 5'-AACACACTGGCTGCATGC-3' (Li et al., 2007).

2.8. Transient transfection and luciferase assay

Transient transfection of freshly isolated CD4T cells from both WT and CIITA^{Tg} cells were performed as described (Sisk et al., 2003). Actin and NF- κ B promoter driven luciferase constructs were described in (Nickerson et al., 2001). Cell lysates were prepared to assess the luciferase activity. Luciferase activity in each group was normalized by protein concentration. The results were expressed as relative luciferase activity (RLA) compared with the value obtained from empty vector-transfected cells.

3. Results

3.1. IL-6 production by CD4 T cells influences Th2 cell differentiation

To investigate the underlying mechanisms for Th2 cytokine expression in T-CD4 T cells, we examined IL-6, since it is produced by multiple cell types can induce IL-4 expression in CD4 T cells (Diehl and Rincon, 2002). For this study, we used total splenic CD4 T cells from wild type control (WT hereon) mice that only have E-CD4 T cells. As a source of T-CD4 T cells, we used CIITA^{Tg} mice (Tg hereon) that generate T-CD4 T cells together with E-CD4 T cells (Li et al., 2005). Because a cell surface marker that can distinguish T-CD4 T cells from E-CD4 T cells is not yet available, we used total CD4 T cells from Tg mice. Our previous studies have shown that CD4 T cells from Tg mice exhibit a distinct phenotype from WT CD4 T cells, even though Tg CD4 T cells contain both E- and T-CD4 T cells (Li et al., 2007).

Enriched CD4 T cells were cultured under the non-skewing (Th0), Th1, or Th2 inducing condition as described in Section 2. As a stimulus, we used plate-bound anti-CD3 instead of APC to eliminate the effect of IL-6 produced by APC. Five days later, cells were then re-stimulated with anti-CD3 overnight and the levels of cytokines in the supernatants were measured by ELISA. We found that WT CD4 T cells stimulated under the Th0 but not Th1 condition produced IL-4 and CD4 T cells from Tg mice produced IL-4 under Th0 and Th1 conditions (Fig. 1A top panels). When the amount of IL-6 was compared, the level was higher in the Tg CD4 T cell culture than WT CD4 T cells (Fig. 1A bottom panels). Th1 cultures of both WT and Tg CD4 T cells made high and comparable levels of IFN- γ (data not shown).

To test whether elevated IL-4 levels are caused by enhanced IL-6, we differentiated enriched splenic CD4 T cells in the presence of a neutralizing antibody against IL-6 under the Th1 or Th2 condition for 5 days. Cells were then restimulated with PMA and ionomycin for 5 h followed by intracellular cytokine staining (ICS) to assess IL-4 and IFN- γ production. WT Th1 cells showed a small reduction in IL-4 positive cells (Fig. 1B, top left group). In contrast, the IL-4 producing population of Tg cells was substantially decreased in the Th1 culture when the antibody was present (Fig. 1B, top right group). The results from three independent experiments showed the greater inhibitory effect of IL-6 neutralization for Tg cells than WT cells (Fig. 1C). We also observed that the neutralization of IL-6 enhanced IFN- γ production slightly, which could be due to reduced endogenous IL-4 production. In contrast, neutralizing IL-6 during Th2 differentiation did not show an effect (Fig. 1B, bottom panels). This is most likely due to the fact that exogenously added IL-4 is a potent inducer of Th2 overriding the effect of IL-6 neutralization. Therefore, we focused on Th1 cells for the subsequent experiments.

We have reported that the proportion of effector/memory-like CD4 T cells were increased in Tg mice compared to WT mice as determined by CD44 expression (Li et al., 2005). Therefore, the results shown above could be explained by more effector cells in Tg than WT cell cultures. If so, IL-4 made by effector cells could be responsible for the induction of IL-4 expression of uncommitted neighboring CD4 T cells. To address this, we sorted naïve CD4 T cells (CD45RB^{hi}CD44^{lo}) and differentiated them under the Th0 or Th1 condition with or without the anti-IL-6 antibody. Naïve cells from Tg mice also showed a significant decrease of IL-4 production by neutralizing IL-6 under both Th0 (Fig. 1D) and Th1 (Fig. 1E) conditions. The decrease in IL-4 protein by the anti-IL-6 antibody treatment was due to the reduced IL-4 mRNA as shown in Fig. 1F. Taken together, elevated IL-6 production by T-CD4 T cells seems to be responsible for the Th2-like cell phenotype.

3.2. PKC θ participates in increased IL-4 expression in Tg CD4 T cells

Previously, PKC θ has shown to play a role in IL-6 production in colonic lamina propria CD4 T cells (Hokama et al., 2004). Furthermore, inhibition of PKC θ activity by using the PKC θ inhibitor rottlerin (RT) or the deficiency of PKC θ resulted in the impairment of IL-6 induction by galectin-4, an epithelial lectin (Hokama et al., 2004). Moreover, PKC $\theta^{-/-}$ CD4 T cells can be differentiated into Th1 cells but are compromised in Th2 development (Marsland et al., 2004). Therefore, we asked whether PKC θ is also involved in IL-6-mediated production of IL-4. To do this, we first examined phosphorylation of PKC θ , which is indicative of PKC θ activation. CD4 T cells were enriched and lysed immediately or after stimulation with anti-CD3 or anti-CD3 together with anti-CD28 antibody for 15 min. Whole cell lysates from the equal number of cells were used for immunoblot. We found that the level of phosphorylated PKC θ (p-PKC θ) prior to stimulation was substantially higher in Tg CD4 T cells compared to WT CD4 T cells (Fig. 2A, compare lanes 1 and 4). Stimulated Tg cells also showed slightly increased pPKC θ . This suggests that constitutively high PKC θ activity in Tg CD4 T cells may induce IL-6, resulting in the induction of IL-4 production. If this is the case, modulating PKC θ activity could affect IL-4 expression in Tg Th1 cells.

To test this hypothesis, enriched CD4 T cells from WT and Tg mice were differentiated under the Th1 condition in the presence or absence of the PKC θ specific inhibitor RT for 5 days and cells were then restimulated overnight by anti-CD3. The concentration of RT (0.5 μ M/ml) used for the experiment was not toxic to cells as cell viability was not affected by RT (data not shown). As shown in Fig. 2B, IL-6 production was reduced by adding RT. Moreover, we observed a decrease in IL-4 as well as IL-5 and IL-13 in the RT-treated culture (Fig. 2C). However, RT did not change the IFN- γ level (Fig. 2C), indicating that the RT-mediated inhibition is not a global inhibitory effect by a pharmacological reagent. Rather, the effect of PKC θ inhibition appears restricted to Th2 cell differentiation.

We also examined the effect of RT on the RNA levels of IL-4 and IFN- γ . Similar to the effect of neutralizing IL-6, IL-4, but not IFN- γ , RNA was less abundant when RT was present (Fig. 2D). One of the hallmarks of active transcription of genes including the IL-4 gene is histone acetylation (Avni et al., 2002). To determine whether the IL-4 locus is differentially acetylated in T-CD4 T cells, we performed a chromatin immunoprecipitation assay. As demonstrated in Fig. 2E, Tg CD4 T cells expressing more IL-4 showed elevated histone acetylation at the internal enhancer region of the

IL-4 locus (compare lanes 1 and 3). However, upon RT treatment, the amount of histone acetylation at the same region was decreased (Fig. 2E, compare lanes 3 and 4). The results suggest that PKC θ participates in chromatin remodeling of the IL-4 locus, either directly or indirectly via IL-6-mediated signaling.

3.3. PKC θ plays a role during the early stage of CD4 T cell differentiation

It has been shown that CD4 T cells become polarized in 2 days upon receiving either the Th1 or Th2 differentiation signal although the full commitment takes several days (Mosmann et al., 1986). Because inhibiting PKC θ by RT efficiently blocked Th2 cytokine production by Tg Th1 cells, we asked when PKC θ exerts its effect during Th cell differentiation.

To determine the time window, we added RT at day 0, 1, or 2 during Th1 differentiation (Fig. 3A). Cells were then cultured and restimulated, and the cytokine levels were compared between WT and Tg Th1 cells. We first assessed the level of IL-6. A drastic inhibition of IL-6 production was observed when RT was introduced at the beginning of differentiation (Fig. 3B and C). However, the effect was gradually diminished as RT was added at later time points, and the inhibition of PKC θ did not show an effect when cells had undergone 2 days of differentiation. The pattern of IL-4 production was very similar to IL-6. Again, IFN- γ levels were comparable strengthening the significance of PKC θ for IL-6 and IL-4 but not IFN- γ . Together, the results suggest that PKC θ is essential during an early differentiation phase to activate IL-6 expression, which appears to be responsible for the induction of IL-4.

3.4. MAPK signaling does not contribute to Th2 cytokine production by Tg CD4 T cells

As shown in Fig. 2A, freshly isolated CD4 T cells from Tg mice showed that PKC θ was highly phosphorylated prior to stimulation. Therefore, we asked whether downstream signaling events might also have been altered, which in turn favors to lead Th2 cytokine gene expression. Mitogen-activated protein kinase (MAPK) are signaling intermediates known to be important for regulating T helper cell differentiation as well as downstream effector molecules of PKC θ . TCR-induced ERK activation controls early IL-4 expression in naïve CD4T cells (Jorritsma et al., 2003). In addition, JNK participates in Th cell development. Mice deficient in JNK1 show preferential differentiation to the Th2 phenotype (Dong et al., 2000), where as JNK2 knockout mice exhibit diminished IFN- γ production, resulting in the impairment of Th1 differentiation (Yang et al., 1998). In addition, p38 MAPK plays an important role in T helper cell differentiation (Platanias, 2003). Therefore, we first assessed the phosphorylation status of MAPK in WT and Tg CD4 T cells.

An equal number of Tg and WT CD4 T cells were stimulated with anti-CD3 alone or anti-CD3 together with anti-CD28 antibodies for 15 min. Whole cell lysates were then used for immunoblots. As shown in Fig. 4A, WT and Tg cells show comparable amount of MAPK phosphorylation prior to, and after antigen-receptor stimulation. To further determine whether ERK or JNK is necessary for the induction of IL-4 by IL-6, we used pharmacological reagents that inhibit MAPK pathways with different specificity: U0126 and SP600125, selectively inhibiting MEK1/2 and JNK activation, respectively. Total CD4 T cells were differentiated under the Th1 condition with or without the inhibitor followed by ICS to measure IL-4 and

IFN- γ production. Blocking JNK with SP600125 showed a marginal effect on IL-4 and IFN- γ (Fig. 4B, second panels). In contrast and as reported, inhibiting MEK1/2 induced IL-4 in WT cells (Fig. 4B, top third panel). Tg cells also exhibited a similar pattern of response suggesting it is unlikely that MAPK contributes to Th2 cytokine production by Tg Th1 cells (Fig. 4B, bottom panel).

3.5. NF- κ B activity is enhanced in Tg CD4 T cells

PKC θ plays a critical role in activation of NF- κ B when T cells are stimulated by TCR together with CD28 as demonstrated in PKC θ deficient cells (Coudronniere et al., 2000). In addition, activation of NF- κ B is necessary to induce IL-6 gene expression in T cells (Libermann and Baltimore, 1990). Together, it is possible that IL-6-mediated induction of IL-4 also depends on NF- κ B. To test this, CD4 T cells were differentiated under the Th1 condition in the presence or absence of SN50 that prevents the nuclear localization of p50 subunit of NF- κ B (Das et al., 2001). After 5 days of differentiation, the cells were re-stimulated with anti-CD3 and the amount of IL-6, IL-4 and IFN- γ was measured. As shown in Fig. 5A, the level of IL-6 was decreased in the culture with SN50 confirming the role of NF- κ B in IL-6 induction. Moreover, when the same supernatants from the culture were used to assess IL-4, inhibition of NF- κ B also affected IL-4 production (Fig. 5A). Again, IFN- γ levels did not show a measurable difference with or without SN50. We also performed intracellular staining of cytokines and found that the data are comparable to those of ELISA (Fig. 5B). To further test the NF- κ B activity, we transiently transfected a luciferase reporter driven by the NF- κ B promoter to WT or Tg CD4 T cells. Tg cells exhibited the greater luciferase activity in Tg than WT cells (Fig. 5C), suggesting enhanced NF- κ B induces IL-6 expression, which directs IL-4 production in Tg cells.

4. Discussion

Our current study highlights the significance of IL-6 made by CD4 T cells that contributes to the Th cell differentiation process. Although IL-6 provided by APC can induce IL-4 expression in CD4 T cells (Rincon et al., 1997), our study revealed that IL-6 produced by CD4 T cells is sufficient to drive transcription of the IL-4 gene in the absence of exogenously provided IL-6 or IL-4. Tg CD4 T cells produced a high level of IL-6 and blocking IL-6 resulted in reduced IL-4 production. The effect of IL-6 was particularly pronounced under the Th1 differentiation condition. Tg CD4 T cells can produce high levels of Th2 cytokines under the Th1 condition but blocking IL-6 greatly diminished the production of these cytokines. Although WT cells showed a smaller effect of IL-6 on IL-4 production than Tg CD4 T cells, the role of IL-6 contributing to Th2 cells was clearly demonstrated. Once these cells produce IL-4, they may be able to amplify Th2 cell differentiation, which will then generate a mixed effector cell phenotype during an immune response. This could then lead to switching of an immune response from Th1 to Th2 immunity unless there is a strong Th1 inducing stimulus. Unimmunized Tg mice have elevated IgE in serum (Li et al., 2007), suggesting that this type of immunity is prominent in Tg mice. It is also reported that IL-6 inhibits Th1 differentiation by a negative feedback mechanism (Diehl et al., 2000). Together, T cell activation seems to drive CD4 T cells towards a Th2 lineage by expressing IL-6, unless the local cytokine environment favors them to differentiate to Th1 cells. Perhaps, this mechanism is beneficial to a host to prevent unnecessary activation of a Th1 response leading to cell-mediated immunity, which could be more detrimental than a Th2 response.

Studies have shown that PKC θ is essential for Th2 cytokine (Marsland et al., 2004). PKC θ deficient mice are impaired in mounting Th2 cell immune responses as demonstrated in experimental models (Marsland et al., 2004). However, PKC θ deficiency does not alter Th1 immune responses supporting the role of PKC θ in Th2 but not Th1 cell development. When

CD4 T cells lacking PKC θ were stimulated *in vitro* under the Th2 condition, they failed to produce Th2 cytokines (Marsland et al., 2004). Previous reports have shown that galectin-4-mediated production of IL-6 from intestinal lamina propria CD4 T cells is induced by PKC θ associated pathway through immunological synapse (Hokama et al., 2004). Therefore, the high expression of IL-6 correlates with hyper-phosphorylation of PKC θ and neutralizing the activity of PKC θ blocks IL-6 expression and subsequent IL-4 production is severely effected. However, adding IL-6 did not rescue IL-4 expression when PKC θ activity was compromised (Sofi and Chang, unpublished data). The underlying mechanisms for this observation are not yet clear but PKC θ seems to regulate IL-4 expression in an IL-6-dependent and -independent manner. Nevertheless, neutralizing PKC θ activity affects the IL-4 chromatin remodeling suggesting that the effect is at the transcription level. However, the effect of neutralizing IL-6 during Th2 cell differentiation was not observed. This is probably due to the potency of IL-4 added to the Th2 culture that can override the IL-6 dependency.

Several studies have reported the role of NF- κ B in Th cell differentiation (Das et al., 2001). Inhibition of the p50 subunit of NF- κ B diminished Th2 cytokine expression, whereas the same subunit did not appear to be involved in the expression of IFN- γ suggesting a specific role of NF- κ B in Th2 but not Th1 cytokine gene expression (Das et al., 2001). Our data further support the involvement of NF- κ B in IL-4 expression. Increased NF- κ B activity correlates with hyperphosphorylation of PKC θ and increased IL-6 expression. Reports have shown that the regulatory regions in the IL-4 gene do not have an NF- κ B binding site (Das et al., 2001). Based on our present study, NF- κ B-mediated activation of IL-4 is likely mediated by IL-6.

Freshly isolated CD4 T cells from Tg mice showed highly phosphorylated PKC θ , which appears to contribute to IL-4 production. Currently, it is not clear what triggers spontaneous PKC θ phosphorylation in Tg mice. Phosphorylation of other signaling molecules including AKT, Stat5, Stat4, Src, SHIP2 and PTEN were not grossly different between WT and Tg CD4 T cells (Sofi and Chang, unpublished data). Further studies are warranted to understand the relationship between T cell activation and IL-6-mediated induction of IL-4 production. Nevertheless, histone H3 and H4 acetylation at the IL-4 intronic enhancer is enhanced in both CD4 SP thymocytes and naïve CD4 T cells from Tg mice (Li et al., 2007). Moreover, IL-4 expression in Tg CD4 T cells occurs in the absence of Stat6 (Li et al., 2007), suggesting that the IL-4 locus is already remodeled by the time CD4 T cells reach to the peripheral lymphoid tissues. If so, CD4 T cells are poised to secrete Th2 cytokines upon activation without going through chromatin remodeling of the IL-4 locus. Consistent with this notion, Tg CD4 T cells can produce IL-5 and IL-13 without IL-4 (Patel et al., 2005). Interestingly, a study has reported that IL-6 induced c-Maf expression in an IL-4 and Stat6-independent manner (Yang et al., 2005). Instead of Stat6, Stat3 binds to the c-Maf promoter and activates the expression (Yang et al., 2005). Therefore, Stat3 that is activated by IL-6R ligation links IL-6 to c-Maf that is a transcription factor that selectively regulates the IL-4 gene. Therefore, IL-6-mediated induction of IL-4 locus is operated using a distinct mechanism that does not involve Stat6. It would be interesting to test whether the c-Maf deficiency would compromise IL-4 production by IL-6. Although NFATc is known to be induced by IL-6 (Yang et al., 2005), it is not clear whether NFAT activation by IL-6 requires Stat3 or not.

In summary, we have shown that IL-6 production by CD4 T cells induces IL-4 in an autocrine manner, which requires PKC θ , NF- κ B. Our study provides a new insight into the molecular mechanism that regulates Th2 cell differentiation.

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Fig. 1.

IL-6 produced by CD4 T cells induces IL-4. (A) Enriched CD4 T cells were differentiated under Th0, Th1 and Th2 inducing conditions as described in Section 2. Cells were restimulated with anti-CD3 overnight and the supernatant were analyzed for IL-4 and IL-6. (B) CD4 T cells were differentiated under Th1 and Th2 inducing conditions in presence and absence of anti-IL-6 neutralizing antibody (1 μ g/ml). Cells were re-stimulated 5 h with PMA and ionomycin and analyzed by intracellular staining. (C) Comparison of IL-4 (left) and IFN- γ (right) expression. Values are mean $\pm$ S.D. of cells in the indicated culture ($n = 3$). (D and E) Naïve CD4 T cells were sorted and differentiated under Th0 (D) and Th1 (E) inducing conditions in presence and absence of anti IL-6 neutralizing antibody at (1 μ g/ml). Cells were restimulated with anti CD3 overnight and the supernatant were analyzed for IL-4 and IFN- γ . (F) CD4 T cells were cultured as in (B). RNA was isolated on day 5 and analyzed by RT-PCR for IL-4, IFN- γ and HPRT expression. Data shown are representative of at least two independent experiments.

Fig. 2.

PKCθ activity is enhanced in Tg CD4 T cells. (A) Western blot was performed using whole cell lysates prepared from freshly isolated CD4 T cells from both WT and Tg mice. The membrane was probed with the antibody detecting p-PKCθ, stripped, and then reprobed with anti-PKCθ. (B and C) Enriched CD4 T cells from WT and Tg mice were differentiated to Th1 condition for 5 days in the presence or absence of PKCθ specific inhibitor RT (0.5 μM). Cells were restimulated with anti-CD3 overnight, and supernatants were analyzed for IL-6 (B) and IL-4, IL-5, IL-13 and IFN-γ (C). (D) Th1 differentiated cells were prepared as in (B). These cells on day 5 were used for RNA isolation and were analyzed by RT-PCR for IL-4, IFN-γ and HPRT expression. (E) Cells were prepared as in (B). These cells at day 5 were used for ChIP assay with anti-acetylated histone H3 antibody. No antibody was used as the negative control for the immuno precipitation and primers specific CD3ε were used as an internal loading control. Data shown are representative of at least two independent experiments.

Fig. 3. PKC θ acts during the initial stage of Th cell differentiation. (A) Schematic diagram illustrating experimental protocol. (B) RT was added at different time points as indicated to the Th1 cultures using enriched CD4 T cells from WT and Tg mice. Cells were then restimulated with anti-CD3 overnight and supernatants were analyzed by ELISA. (C) Cells described in (B) were re-stimulated for 5 h with PMA and ionomycin followed by intracellular cytokine staining. Data shown are representative of at least three independent experiments.

Fig. 4.

Marginal role of MAPK in IL-6-mediated induction of IL-4. (A) An equal number of freshly isolated CD4T cells from WT and Tg mice were kept unstimulated or stimulated with anti-CD3 alone or anti-CD3 together with anti-CD28 for 15min. Whole cell lysates were prepared and used for western blotting with the indicated antibody. Data shown are representative of at least three independent experiments. (B) Enriched CD4T cells were prepared from WT and Tg mice and cultured under the Th1 condition in the presence of MAPK specific inhibitors (0.5 μ M/ml and 1 μ M/ml for ERK and JNK activity inhibition respectively) as shown. Cells were re-stimulated for 5 h with PMA and ionomycin followed by intracellular cytokine staining. Data shown are representative of at least two independent experiments.

Fig. 5.

IL-4 induction by IL-6 depends on NF-κB p50 subunit. (A) Enriched CD4 T cells from WT and Tg were pretreated with 100 μg of SN50 for 1 h and then stimulated under the Th1 inducing condition. Cells were then re-stimulated with anti-CD3 overnight and supernatants were collected to analyze the amount of cytokines by ELISA. (B) Cells were prepared as in (A) and re-stimulated for 5 h with PMA and ionomycin for intracellular staining analysis. (C) Enriched CD4 T cells were stimulated overnight and then transfected with the luciferase reporter driven by the actin or the NF-κB promoter. The luciferase reporter without the promoter was transfected as a control. Luciferase activity in each group was normalized by the protein concentration. The results were expressed as relative luciferase activity (RLA) compared with the value obtained from the control transfected cells. Data shown are representative of at least two independent experiments.