



Contents lists available at [SciVerse ScienceDirect](#)

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



CD4⁺ Th2 cells function alike effector Tr1 and Th1 cells through the deletion of a single cytokine IL-6 and IL-10 gene

Manjunatha Ankathatti Munegowda^a, Shuling Xu^a, Andrew Freywald^b, Jim Xiang^{a,*}

^a Cancer Research Unit, Research Division, Saskatchewan Cancer Agency, Departments of Oncology and Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

^b Cancer Research Unit, Research Division, Saskatchewan Cancer Agency, Department of Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

ARTICLE INFO

Article history:

Received 29 December 2011
Received in revised form 17 February 2012
Accepted 21 February 2012
Available online xxx

Keywords:

Th2
Th1
Tr1
IL-10
IL-6
CTL

ABSTRACT

Depending on polarizing cytokine signals during activation by antigen, naïve CD4⁺ T cells can be stimulated and differentiated into distinct functional CD4⁺ T cell subsets such as Th1, Th2 and Tr1 cells. Among them, Th2 cells are pathogenic in allergic diseases such as asthma, which are characterized by transcription factor GATA3 expression and IL-4, IL-5, IL-6, and IL-10 cytokine secretion. The overlapping expression of some signature cytokines by Th2 and other subsets of CD4⁺ T cells may not only indicate the plasticity of CD4⁺ T cells, but could also suggest the possibility of the deletion of a single signature cytokine gene leading to the functional differentiation of naïve CD4⁺ T cells into effector Th1 or Tr1 cells under Th2 differentiation conditions. In this work, we stimulated naïve CD4⁺ T cells derived from OT II mice or OT II mice that were deficient in individual cytokines (IL-4, IL-5, IL-6 and IL-10) with OVA-pulsed dendritic cells (DC_{OVA}) in the presence of IL-4 and anti-IFN- γ , to generate OVA-specific wild-type (WT) Th2, and Th2(IL-4 KO), or Th2(IL-5 KO), or Th2(IL-6 KO), or Th2(IL-10 KO) cells, and to assess their capacity in modulating DC_{OVA}-induced CD8⁺ cytotoxic T lymphocyte (CTL) responses, and antitumor immunity in WT C57BL/6 mice. We conclusively demonstrate that GATA-3-expressing Th2 cells enhance DC_{OVA}-induced CTL responses via IL-6 secretion. We also show that IL-6 and IL-10 gene deficient Th2(IL-6 KO) and Th2(IL-10 KO) cells, but not IL-4 and IL-5 gene deficient Th2(IL-4 KO) and Th2(IL-5 KO) cells, behave like functional Tr1 and Th1 cells by inhibiting and enhancing DC_{OVA}-induced OVA-specific CD8⁺ CTL responses and antitumor immunity, respectively. We further elucidate that inhibition and enhancement of DC_{OVA}-induced OVA-specific CTL responses by Th2(IL-6 KO) and Th2(IL-10 KO) cells are mediated by their immune suppressive IL-10 and pro-inflammatory IL-6 secretion, respectively. Taken together, our study suggests that deletion of a single cytokine gene IL-6 and IL-10 makes CD4⁺ Th2 cells become effector CD4⁺ Tr1- and Th1-like cells, respectively. Our data thus not only provide new evidence for another type of CD4⁺ T cell plasticity, but also have a potential to impact the development of a new direction in immunotherapy of allergic diseases.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

In response to infections, various types of immune cells are involved in both innate and adoptive immune responses, and are activated to control and eliminate the invading pathogens. Among them, CD4⁺ T cells play an important role in the defensive immunity. These lymphocytes activate macrophages to develop enhanced microbicidal activity and recruit neutrophils, eosinophils and basophils to sites of inflammation via their secreted cytokines or chemokines thus assisting the innate immunity. In addition, they also help B cells to produce antibodies and license dendritic cells (DCs) to modulate different types of CD8⁺ cytotoxic T lymphocyte

(CTL) immune responses in adoptive immunity (Skapenko et al., 2005).

Depending on polarizing cytokine signals during activation by antigen (Ag), naïve CD4⁺ T cells can initiate various differentiation programs that lead to the development of distinct functional CD4⁺ T cell subsets. Key transcriptional factors act as lineage-specifying regulators coordinating expression of specific cytokine genes (Wilson et al., 2009). For example, the transcriptional factor T-box-containing protein expressed in T cells (T-bet) controls type 1 T help (Th1) cell differentiation program associated with the production of Th1 signature cytokine IFN- γ required for efficient immune responses against intracellular pathogens (Szabo et al., 2000) and tumors (Goedegebuure and Eberlein, 1995). In contrast, GATA-binding protein 3 (GATA-3) is a regulator of the development of type 2 T help (Th2) cells that express IL-4, IL-5, IL-6 and IL-10. These signature cytokines are critical for the immunity against helminthes and other extracellular pathogens (Zheng

* Corresponding author at: Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada. Tel.: +1 306 6552917; fax: +1 306 6552635.
E-mail address: jim.xiang@saskcancer.ca (J. Xiang).

and Flavell, 1997) or for antibodies production (Carter and Dutton, 1996). Unfortunately, Th2 cells are also pathogenic in allergic diseases such as asthma (Gordon et al., 2005). Another subset of CD4⁺ T cells is the Foxp3⁺ IL-10- and IFN- γ -secreting CD4⁺ type 1 regulatory T (Tr1) cells that are generated from naive T cells in the periphery after encounter with Ag presented by DCs in the status distinct from those for promoting the differentiation of Th1 and Th2 cells (Roncarolo et al., 2006). TGF- β plays a major role in Tr1 differentiation (Chen et al., 2003; Bettelli et al., 2006) and development (Liu et al., 2008) by activation of Smad3 to promote Foxp3 expression (Tone et al., 2008). These CD4⁺ Tr cells, which are specific for pathogen-derived Ags, suppressed immune responses via their signature cytokine IL-10 secretion (Hao et al., 2008).

We previously established an ovalbumin (OVA)-specific animal model using OVA-specific T cell receptor (TCR) transgenic OT II CD4⁺ T cell subsets to study the functional effect and molecular mechanisms associated with different subsets of CD4⁺ T cells (Hao et al., 2008; Umeshappa et al., 2009; Ankathatti Munegowda et al., 2011). In this study, we investigate whether manipulation of a single signature cytokine can differentiate naïve CD4⁺ T cells into functional Th1 and Tr1 cells under Th2 differentiation conditions. To assess the role of specific cytokines, we have generated OVA-specific CD4⁺ Th2 cells by *in vitro* stimulation of naïve CD4⁺ T cells derived from OT II mice or OT II mice with deficiency in individual cytokine (IL-4, IL-6, IL-5 or IL-10) with OVA-pulsed DCs (DC_{OVA}) in the presence of IL-4 and anti-IFN- γ antibody, and then compared their capacities in stimulating or inhibiting OVA-specific CD8⁺ T cell responses, and antitumor immunity induced by DC_{OVA} immunization in wild-type C57BL/6 mice.

2. Materials and methods

2.1. Tumor cells, reagents and animals

The OVA-transfected BL6-10 (BL6-10_{OVA}) cell lines were generated in our laboratory (Xiang et al., 2005). The biotin-labeled antibodies (Abs) specific for CD4 (GK1.5), CD11c (HL3), CD25 (7D4), CD40 (K19), CD69 (H1.2F3) and CD80 (16-10A) were obtained from BD Pharmingen, Mississauga, ON, Canada. The FITC conjugated avidin was obtained from Jackson Immuno Research Laboratory Inc. (West Grove, PA). The anti-H-2K^b/OVAI (pMHC I) Ab was obtained from Dr. Germain, National Institute of Health, Bethesda, MD (Porgador et al., 1997). Recombinant cytokines IL-2, IL-4, GM-CSF and TGF- β were purchased from R&D systems Inc. (Minneapolis, MN). IL-6 (MP5-20F3) neutralizing antibody was purchased from eBiosciences (San Diego, CA) and IL-10 (JE55-2A5) neutralizing antibody was purchased from BD Pharmingen, Mississauga, ON, Canada. The PE-H-2K^b/OVAI tetramer and FITC-anti-CD8 Ab (PK135) were obtained from Beckman Coulter, Mississauga, Ontario, Canada. The OVAI (SIINFEKL) peptide specific for H-2K^b and Mut1 (FEQNTAQP) specific for H-2K^b of irrelevant 3LL lung carcinoma (Xiang et al., 2005) were synthesized by Multiple Peptide Systems (San Diego, CA). The C57BL/6 (B6, CD45.2⁺), OVA-specific TCR-transgenic OT II mice, and IL-4, IL-5, IL-6 and IL-10 gene knock-out (KO) mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, MA). Homozygous OT II/IL-4^{-/-}, OT II/IL-5^{-/-}, OT II/IL-6^{-/-}, and OT II/IL-10^{-/-} mice were generated by backcrossing the designated gene KO mice onto the OT II background for three generations; homozygosity was confirmed by polymerase-chain reaction (PCR) according to Jackson laboratory's protocols.

2.2. Preparation of dendritic cells

Mature bone marrow-derived DCs, expressing high levels of CD11c, CD40, CD80 and pMHC class I, were generated as described

previously (Xiang et al., 2005). Briefly, bone marrow cells were collected from femora and tibiae of wild type C57BL/6 mice and RBCs are depleted with 0.84% ammonium chloride. Obtained cells were plated in DC culture medium (DMEM plus 10% FCS, 20 ng/ml GM-CSF and 20 ng/ml IL-4). On day 3 non-adherent granulocytes, T cells and B cells were gently removed and replaced with medium. Two days later, loosely adherent DC's were dislodged and replated and grown till 6 days. On 6th day non-adherent DCs were harvested and pulsed overnight at 37 °C with 0.1 mg/ml OVA (Sigma, St. Louis, MO) and termed DC_{OVA}. Inhibitory DC_{OVA} was derived expressing high levels of IL-10 as described earlier (Huang et al., 2010). Briefly, bone marrow cells depleted with RBCs were cultured in presence of 20 ng/ml GM-CSF in 10% FCS containing DMEM for 10 days, carefully changing medium every 48 h. After 10 days they were cultured for 3 days in presence of 7.5 ng/ml GM-CSF, 50 ng/ml IL-10 in 10% FCS containing DMEM and loosely bound DCs are harvested on last day and pulsed overnight with 0.1 mg/ml OVA.

2.3. Preparation of OT II CD4⁺ Th2 cells

Naïve OVA-specific CD4⁺ T cells were isolated from OT II mouse spleens. T cells were enriched by passage through nylon wool columns and then CD4⁺ cells were purified by negative selection using anti-mouse CD8 paramagnetic beads (DYNAL Inc., Lake Success, NY) to yield populations that were $\geq 95\%$ CD4⁺/V α 2V β 5⁺ T cells. To generate *in vitro* DC_{OVA}-activated CD4⁺ Th2 cells, CD4⁺ T cells (2×10^5 cells/ml) from OT II mice or designated gene-deleted OT II mice were stimulated for 3 days with irradiated (4000 rad) bone marrow derived DC_{OVA} (1×10^5 cells/ml) in presence of IL-4 (20 ng/ml) and anti-IFN- γ Ab (10 μ g/ml) (R&D Systems, Minneapolis, MN). *In vitro* DC_{OVA}-activated CD4⁺ T cells derived from OT II with respective IL-4, IL-5, IL-6 and IL-10 gene KO mice were referred to as Th2(IL-4 KO), Th2(IL-5 KO), Th2(IL-6 KO) and Th2(IL-10 KO), respectively.

2.4. Preparation of OT II CD4⁺ Th1 cells

Naïve OVA-specific CD4⁺ T cells isolated as explained earlier. To generate *in vitro* DC_{OVA}-activated CD4⁺ Th1 cells, CD4⁺ T cells (2×10^5 cells/ml) from OT II mice were stimulated for 3 days with irradiated (4000 rad) bone marrow derived DC_{OVA} (1×10^5 cells/ml) in presence of IL-2 (20 U/ml), IL-12 (5 ng/ml) and anti-IL-4 Ab (10 μ g/ml) (R&D Systems, Minneapolis, MN).

2.5. Preparation of OT II CD4⁺ Tr1 cells

Naïve OVA-specific CD4⁺ T cells isolated as explained earlier. To generate *in vitro* DC_{OVA}-activated CD4⁺ Tr1 cells, CD4⁺ T cells (2×10^5 cells/ml) from OT II mice were stimulated for 5 days with irradiated (4000 rad) bone marrow derived inhibitory IL-10 secreting DC_{OVA} (1×10^5 cells/ml) in presence of IL-2 (20 U/ml), IL-10 (20 ng/ml) and TGF- β (20 ng/ml) (R&D Systems, Minneapolis, MN).

2.6. Phenotypic characterization of CD4⁺ Th1, Th2 and Tr1 cells

For the phenotypic analyses, the above Th1, Th2 and Tr1 cells were stained with a panel of biotin-conjugated Abs. After washing with PBS, these cells were further stained with R-phycoerythrin (PE)-conjugated avidin and analyzed by flow cytometry. Their culture supernatants were analyzed for cytokine expression using ELISA kits (Endogen, Cambridge, MA), as previously described (Xiang et al., 2005).

2.7. RT-PCR

Total RNA was extracted from Th1, Th2 and Tr1 cells with Qiagen RNeasy purification kit (Qiagen, Mississauga, Ontario, Canada) as per manufacturer's protocols. Qiagen quantitative reverse transcription kit (Qiagen) was used to synthesize cDNA, which was then analysed by semi-quantitative PCR in triplicates.

2.8. *In vivo* CD8⁺ T cell proliferation assay

Wild type C57BL/6 ($n=6$) mice were i.v. immunized with irradiated (4000 rad) DC_{OVA} (0.5×10^6 cells) or DC_{OVA} (0.5×10^6 cells) plus Th1 or Tr1 or Th2 or Th2(KO) (3×10^6 cells), respectively. In another experiment wild type C57BL/6 mice were i.v. immunized with irradiated (4000 rad) DC_{OVA} (0.5×10^6 cells) plus Th2(IL-6 KO) or Th2(IL-10 KO) (3×10^6 cells) along with anti-IL-10 or anti-IL-6 neutralizing antibodies on 0 and 3rd day after immunization, respectively. Six days subsequent to immunization, the mouse blood samples were harvested and stained with PE-H-2K^b/OVAI tetramer and FITC-anti-CD8 Ab (PK135) (Beckman Coulter). The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter) and samples were analyzed by flow cytometry according to the company's protocol.

2.9. *In vivo* CD8⁺ T cell cytotoxicity assay

In *in vivo* cytotoxicity assay, 6 days after immunization, the cohort of above immunized mice were i.v. co-injected with 1:1 ratio of splenocytes labeled with high ($3.0 \mu\text{M}$, CFSE^{high}) and low ($0.6 \mu\text{M}$, CFSE^{low}) concentrations of CFSE and pulsed with OVAI and Mut1, respectively (Xiang et al., 2005). Sixteen hours after target cell delivery, the spleens were removed and residual CFSE^{high} and CFSE^{low} target cells remaining in the recipients' spleens were sorted and analyzed by flow cytometry.

2.10. Animal studies

Wild-type C57BL/6 mice ($n=8$) were i.v. immunized with irradiated (4000 rad) DC_{OVA} (0.5×10^6 cells per mouse) alone or in combination with Th1, Tr1, Th2, Th2(IL-6 KO) and Th2(IL-10 KO) (3×10^6 cells per mouse), respectively. Eight days later, the mice were i.v. challenged with BL6-10_{OVA} (0.3×10^6 cells per mouse) tumor cells. The mice were sacrificed 3 weeks after tumor cell challenge and lung metastatic tumor colonies were counted.

2.11. Statistical analysis

All experiments were tested for statistical significance using unpaired, two tailed, Student's *t*-test. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Phenotypic characterization of OVA-specific CD4⁺ Th1, Th2 and Tr1 cells

To generate different subsets of ovalbumin-specific Th1, Th2 and Tr1 cells, naive OT II mouse CD4⁺ T cells were cultured with OVA-pulsed dendritic cells (DC_{OVA}) expressing CD11c, Ia^b, CD40, CD80 and pMHC I (Fig. 1A) in the presence of subset specific differentiation medium. To phenotypically characterize them, these DC_{OVA}-activated CD4⁺ T cells were analyzed by flow cytometry, RT-PCR and ELISA for assessment of expression of cell surface markers, intracellular transcription factors and cytokine secretion, respectively. We demonstrated that these T cells were positive for CD4, CD25, and CD69 expression (Fig. 1B), indicating that they are

active CD4⁺ T cells. CD4⁺ Th1 cells expressed transcription factor T-bet (Fig. 1C) and secreted IL-2 (2.5 ng/ml), IL-6 (0.9 ng/ml), IFN- γ (3.4 ng/ml) but no IL-4, IL-6 and IL-10 (Fig. 1D), whereas CD4⁺ Th2 cells expressed transcription factor GATA-3 and secreted IL-2 (1.4 ng/ml), IL-4 (1.3 ng/ml), IL-5 (1.5 ng/ml), IL-6 (1 ng/ml), IL-10 (0.4 ng/ml) and undetectable levels of IFN- γ , which are consistent with typical phenotypes of CD4⁺ Th1 and Th2 cells, respectively. In contrast, CD4⁺ Tr1 cells expressed transcription factor Foxp3 and secreted IL-5 (0.5 ng/ml), IL-10 (2.5 ng/ml), IFN- γ (1.9 ng/ml), as we previously described (Hao et al., 2008).

3.2. Th1 and Th2 cells augment whereas Tr1 cells inhibit DC_{OVA}-induced CTL responses

To assess the functional effect of different CD4⁺ T subsets on DC_{OVA}-induced CTL responses, DC_{OVA} were injected intravenously into wild type C57BL/6 mice. DC_{OVA}-immunized mice showed OVA-specific CD8⁺ T cell proliferation accounting for 0.79% of the total CD8⁺ T cell population in the peripheral blood (Fig. 2A). When Th2 cells were co-injected with DC_{OVA}, OVA-specific CD8⁺ T cells were increased to 0.97% ($p < 0.05$), indicating that *in vitro* generated Th2 cells enhance DC_{OVA}-induced CTL responses (Fig. 2A). To assess the functional effect of activated CTLs, we adoptively transferred OVAI peptide-pulsed wild-type (WT) C57BL/6 mouse splenocytes strongly labeled with CFSE (CFSE^{high}) and equal proportion of control peptide Mut1 pulsed wild type splenocytes weakly labeled with CFSE (CFSE^{low}) to wild type C57BL/6 mice that were immunized 6 days earlier with DC_{OVA} alone or co-injected with DC_{OVA} and Th1 or Th2 or Tr1 cells. Sixteen hours after their transfer, we found that 50.3% of CFSE^{high} labeled cells were killed and none of the CFSE^{low} labeled cells were killed in DC_{OVA}-immunized mice, whereas in Th2 and DC_{OVA} co-injected mice 68.7% of CFSE^{high} cells were killed (Fig. 2B). In concurrence with CD8⁺ T cell proliferative responses (Fig. 2A), there was significantly increased cytotoxicity associated with Th2 co-injection with DC_{OVA} ($p < 0.05$), indicating that Th2-enhanced CTLs are functional effectors with killing activity to OVA-specific target cells.

3.3. IL-6 gene deficient CD4⁺ Th2 cells behave like functional Tr1 cells by inhibiting DC_{OVA}-induced CTL responses in an IL-10 dependent manner

IL-6 gene deficient CD4⁺ Th2 [Th2(IL-6 KO)] cells were obtained by culturing DC_{OVA} with naïve CD4⁺ T cells derived from OTII/IL-6^{-/-} mice in the presence of IL-4 and anti-IFN- γ Ab. These Th2(IL-6 KO) cells had similar phenotypes, including expression of cell surface markers and transcription factor, as WT Th2 cells (data not shown). In addition, while being IL-6 deficient, they secreted IL-2 (0.5 ng/ml), IL-4 (0.6 ng/ml), IL-5 (1.1 ng/ml), IL-10 (2.5 ng/ml), IFN- γ (1.4 ng/ml) (Fig. 3A). Interestingly, we found that DC_{OVA}-induced OVA-specific CTL responses dramatically declined from the original 0.79% to only 0.18% (Fig. 3B). In contrast, control, IL-4 gene deficient CD4⁺ Th2 [Th2(IL-4 KO)] cells and IL-5 gene deficient CD4⁺ Th2 [Th2(IL-5 KO)] cells did not modulate DC_{OVA}-stimulated OVA-specific CTL responses. To elucidate the molecular mechanism underlying immune suppression by Th2(IL-6 KO) cells, we i.v. injected mice with neutralizing anti-IL-10 Ab simultaneously with Th2(IL-6 KO) cells to block IL-10 signal. We found anti-IL-10 Ab to completely inhibit the immune suppressive effect of Th2(IL-6 KO) cells on DC_{OVA}-induced OVA-specific CTL responses (Fig. 3C), indicating that Th2(IL-6 KO) cells behaving in analogy to Tr1 cells suppress immune responses by secretion of IL-10 cytokine, which is consistent with our previously published observation (Hao et al., 2008).

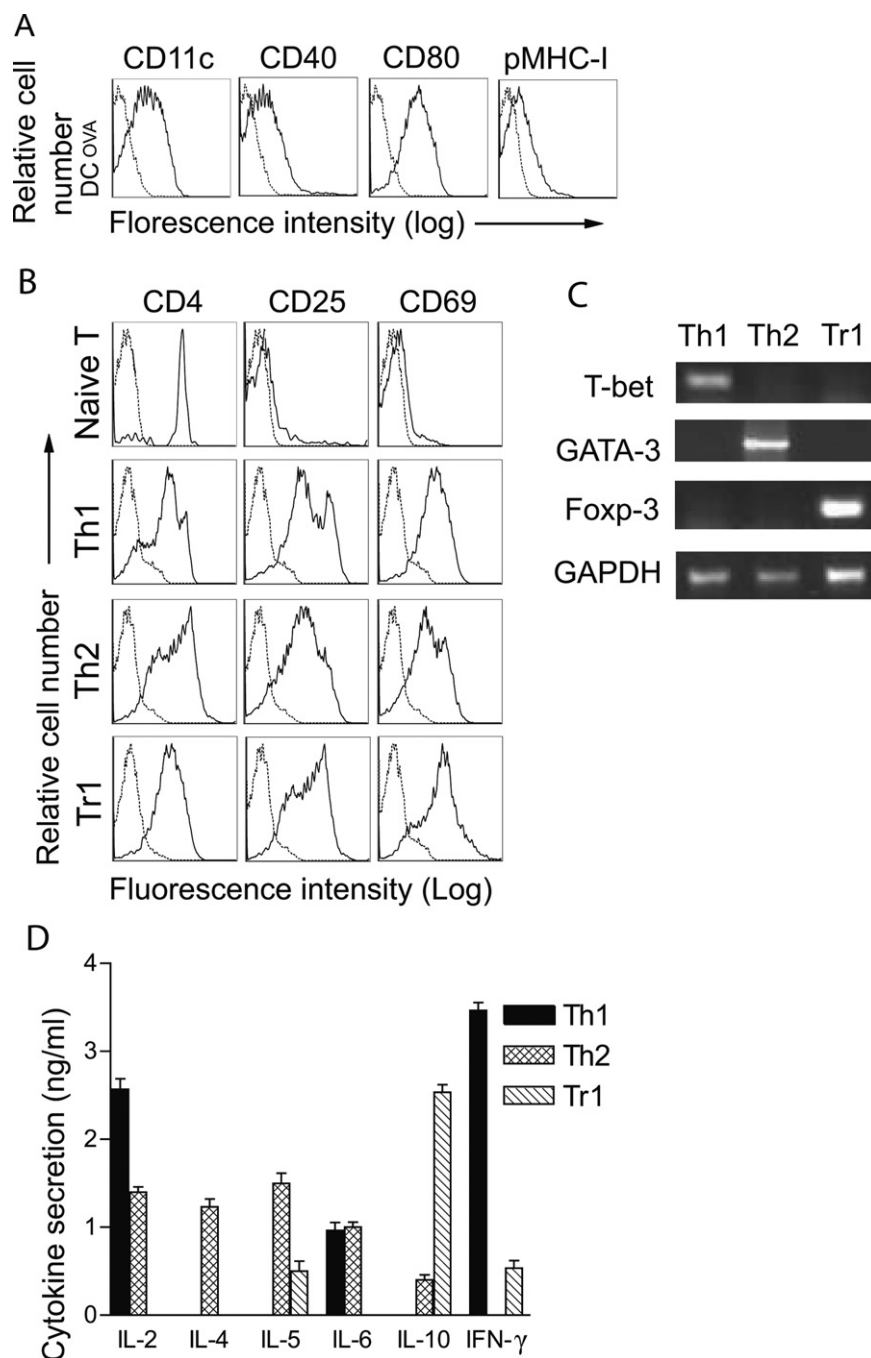


Fig. 1. Phenotypic characterization of Th1, Th2, Tr1 and DC_{OVA}. (A) Flow cytometric analysis of DC_{OVA}. *In vitro* generated DC_{OVA} were stained with panel of Abs for analysis of cell surface expression of CD11c, CD40, CD80 and pMHC-I (thick solid lines). Isotype-matched irrelevant Abs were used as controls (dotted lines). (B) Flow cytometric analysis. *In vitro* DC_{OVA}-activated Th1 and Th2, and *in vitro* inhibitory DC_{OVA} activated-Tr1 population were stained with a panel of Abs for analysis of cell surface expression of CD4, CD25 and CD69 (thick solid lines). Isotype-matched irrelevant Abs were used as controls (dotted lines). (C) RNA extracted from Th1, Th2 and Tr1 cells were analyzed by RT-PCR to assess the expression of T-bet, GATA-3 and Foxp-3. (D) The supernatants of Th1, Th2 and Tr1 cells were assayed for IL-2, IL-4, IL-5, IL-6, IL-10 and IFN- γ secretion by ELISA. One representative experiment of three is displayed.

3.4. IL-10 gene deficient CD4⁺ Th2 cells behave like functional Th1 cells by enhancing DC_{OVA}-induced CTL responses through IL-6 cytokine

IL-10 gene deficient CD4⁺ Th2 [Th2(IL-10 KO)] cells were obtained by culturing DC_{OVA} with naïve CD4⁺ T cells derived from OTII/IL-10^{-/-} mice in the presence of IL-4 and anti-IFN- γ Ab. Based on the expression of cell surface markers and of the GATA-3 transcription factor, these Th2(IL-10 KO) cells had a phenotype resembling WT Th2 cells (data not shown). In addition, despite

IL-10 deficiency, they secreted IL-2 (0.5 ng/ml), IL-4 (1.2 ng/ml), IL-5 (2.5 ng/ml), IL-6 (1.5 ng/ml), IFN- γ (2.4 ng/ml) (Fig. 3A). Interestingly, we found that DC_{OVA}-stimulated OVA-specific CTL responses significantly increased from the original 0.97%, triggered by DC_{OVA}, to 1.28% ($p < 0.01$) in the presence of DC_{OVA} and Th2(IL-10 KO) co-stimulation (Fig. 3B), which is similar to enhancement of CTL responses by Th1 cells (Fig. 2A). To elucidate the molecular mechanism underlying the immune enhancement by Th2(IL-10 KO) cells, we co-injected the mice with neutralizing anti-IL-6 Ab and Th2(IL-10 KO) cells to block IL-6 signal. In this experiment,

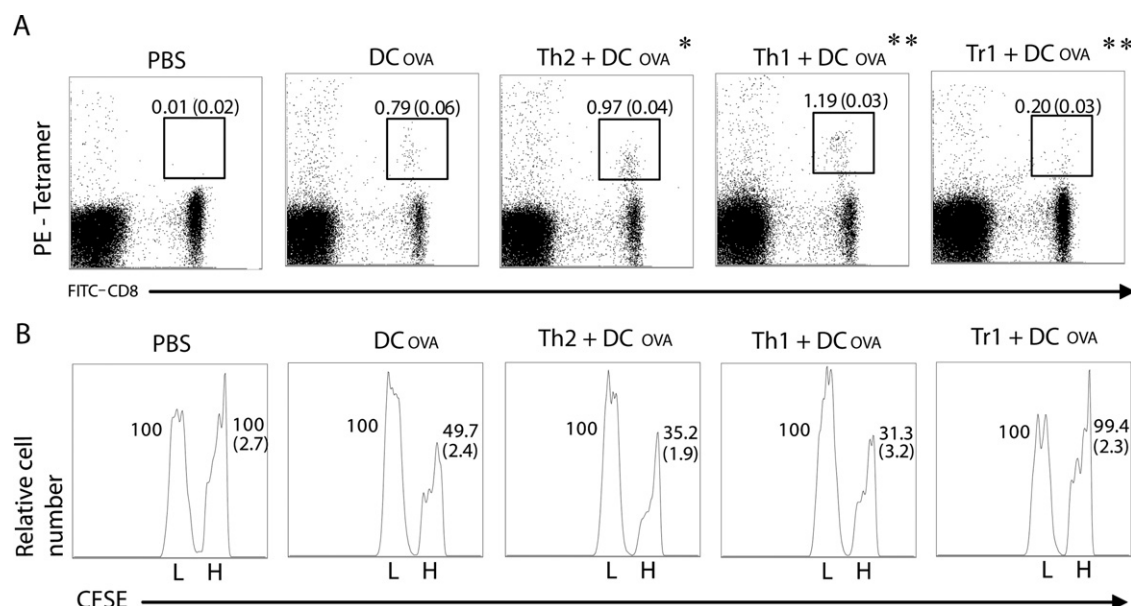


Fig. 2. Stimulatory or inhibitory effects of Th1, Th2 and Tr1 cells on DC_{OVA} induced CD8⁺ T cell response. (A) Tetramer staining assay. Wild type C57BL/6 mice (6 mice per group) were immunized with PBS, DC_{OVA}, DC_{OVA} along with Th1/Th2/Tr1 cells. Six days after immunization tail blood samples of immunized mice were stained with PE-H-2K^b/OVA tetramer and FITC-anti-CD8 Ab, and analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive CD8⁺ T cells versus the total CD8⁺ T cells with standard deviation in parenthesis. The differences between DC_{OVA} and Th2 + DC_{OVA} mice (*) or Th1 + DC_{OVA} and Tr1 + DC_{OVA} mice (**) are significant ($p < 0.05$) or very significant ($p < 0.01$) (Student's *t*-test). (B) *In vivo* cytotoxicity assay. The residual CFSE^{high} (H) and CFSE^{low} (L) target cells remaining in the recipient spleens were analyzed by flow cytometry. The value in each panel represents the percentage of CFSE^{high} versus CFSE^{low} target cells remaining in the spleen with standard deviation in parenthesis. ($n = 6$, average $\pm$ SD). One representative experiment of two is shown.

anti-IL-6 Ab completely blocked the enhancement of DC_{OVA}-induced CTL responses initiated by Th2(IL-10 KO) cells (Fig. 3C), indicating that Th2(IL-10 KO) behave similar to Th1 population and augment immune responses by secreting IL-6 (Table 1).

3.5. IL-6 gene deficient CD4⁺ Th2 cells inhibit and IL-10 deficient cells enhance DC_{OVA}-triggered OVA-specific antitumor immunity

To confirm the above observations that IL-6 gene deficient CD4⁺ Th2 cells suppress, while IL-10 deficient cells promote DC_{OVA}-induced OVA-specific CTL responses, we conducted animal experiments and monitored antitumor immunity. Wild-type C57BL/6 mice were immunized with DC_{OVA} and DC_{OVA} accompanied by Th1, Th2, Tr1 or Th2(KO) cells. All immunized mice were challenged with BL6-10_{OVA} tumor cells 8 days following the immunization. DC_{OVA} immunized mice demonstrated complete protection (8/8) from lung tumor metastasis (Table 2). DC_{OVA}-immunized mice with the co-injection of Th1, Th2 or Th2(IL-10 KO) cells were equally protected (8/8), however mice co-injected with Tr1 or Th2(IL-6 KO) cells all (8/8) died of lung tumor metastasis, confirming that Th2(IL-10 KO) cells act like functional Th1 and Th2(IL-6 KO) behave similar to Tr1 cells in stimulating, and suppressing DC_{OVA}-induced antitumor immunity.

4. Discussion

It is canonically accepted that Th1 and Th2 responses are counter-regulated by one another. Cross-regulation of Th1- and

Th2-specific cytokines has been documented, and Th1 cytokines IFN- γ and IL-12 have been shown to dampen IL-4-mediated granuloma formation in schistosomiasis (Lukacs and Boros, 1993; Oswald et al., 1994; Boros and Whitfield, 1999). Originally, Khoruts et al. (1995) showed that Th2 cells were inefficient in suppression of Th1 cell-mediated experimental autoimmune encephalomyelitis (EAE). Fernando et al. (1998) demonstrated that Th2 cells neither enhanced nor suppressed antitumor CTL responses. Later, however, another group showed Th2 cells to play role in suppressing Th1 responses during helminth infection (McKee and Pearce, 2004). In addition, Th2 cells also inhibited alloantigen-specific delayed-type hypersensitivity (Waaga et al., 2001) and marrow graft rejection (Erdmann et al., 2004). In this study, we generated OVA-specific CD4⁺ T-bet-expressing Th1, GATA-3-expressing Th2 and Foxp3-expressing Tr1 cells by culturing OVA-pulsed DC_{OVA} with OTII CD4⁺ T cells under different culture conditions and assessed their modulatory effect on DC_{OVA}-induced OVA-specific CD8⁺ CTL responses. We demonstrate that OVA-specific CD4⁺ Th1 and Tr1 cells enhance and suppress DC_{OVA}-induced CTL responses, respectively, consistent with our previous reports (Hao et al., 2008). Interestingly, we

Table 2

Inhibitory or stimulatory effects of Th2 cells on DC_{OVA}-induced immunity against lung tumor metastases.

Immunization	Tumor bearing mice (%)	Median number of colonies
DC _{OVA}	0/8 (0)	0
Th2 + DC _{OVA}	0/8 (0)	0
Th1 + DC _{OVA}	0/8 (0)	0
Tr1 + DC _{OVA}	8/8 (100)	>100
Th2(IL-6 KO) + DC _{OVA}	8/8 (100)	>100
Th2(IL-10 KO) + DC _{OVA}	0/8 (0)	0
PBS	8/8 (100)	>100

C57BL/6 mice (8 per group) were i.v. immunized with DC_{OVA} and injected with Th2 or Th1 or Tr1 or Th2 with cytokine gene deficiency. Eight days after injection, mice were challenged with BL6-10_{OVA} cells. The mice were sacrificed 3 weeks after tumor cell challenge and lung metastatic tumor colonies were counted. One representative experiment of two is shown.

Table 1
Polarizing culture conditions of different subsets of T-helper cells.

T-helper subset	Polarizing culture conditions condition
Th1	IL-2 (20 U/ml), IL-12 (5 ng/ml) and anti-IL-4 Ab (10 μ g/ml)
Th2	IL-4 (20 ng/ml) and anti-IFN- γ Ab (10 μ g/ml)
Tr1	IL-2 (20 U/ml), IL-10 (20 ng/ml) and TGF- β (20 ng/ml)

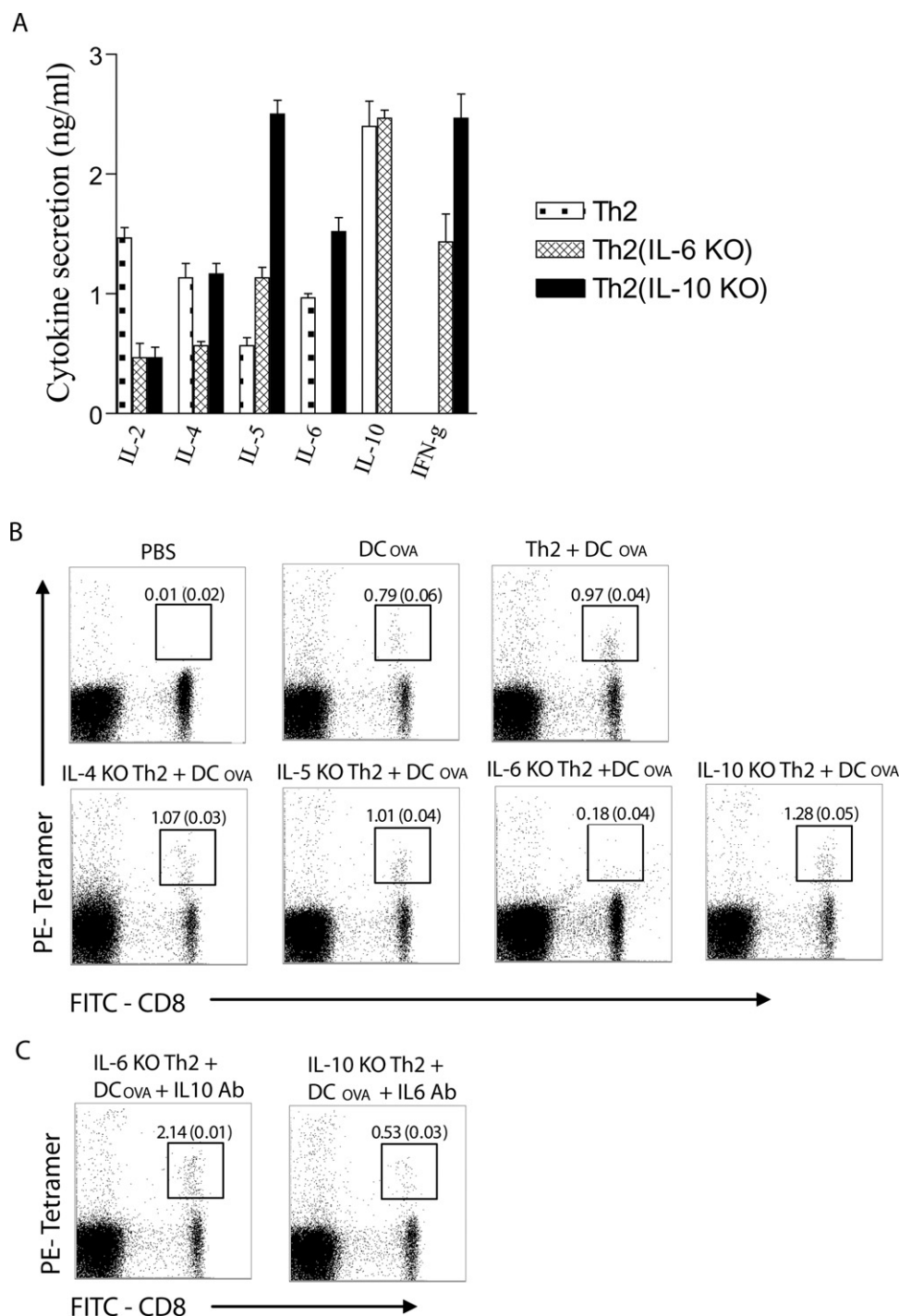


Fig. 3. Stimulatory or inhibitory impacts of cytokine gene deficient Th2 cells on DC_{OVA} induced CD8 $^+$ T cell responses. (A) The supernatants of Th2, Th2(IL-6 KO) and Th2(IL-10 KO) cells were analyzed cytokines IL-2, IL-4, IL-5, IL-6, IL-10 and IFN- γ secretion by ELISA. (B and C) Tetramer staining assay. In experiment B, wild type C57BL/6 mice (6 mice per group) were immunized with DC_{OVA} and different knockout Th2. In experiment C, C57BL/6 mice (6 mice per group) were immunized with DC_{OVA} and different knockout Th2, and simultaneously these mice were injected twice (day 0 and day 3) with depicted neutralizing antibodies. Six days after immunization tail blood samples of immunized mice were stained with PE-H-2K ^{b} /OVAI tetramer and FITC-anti-CD8 Ab, and analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive CD8 $^+$ T cells versus the total CD8 $^+$ T cells with standard deviation in parenthesis ($n = 6$, average $\pm$ SD).

clearly show that OVA-specific CD4 $^+$ Th2 cells also enhance DC_{OVA}-induced CTL responses via inflammatory IL-6 secretion possibly due to the stimulatory effect on T cell proliferation (Takeda et al., 1998) and counteractive effect on CD4 $^+$ CD25 $^+$ Tr cell-mediated inhibition (Pasare and Medzhitov, 2003) mediated by the IL-6 cytokine.

The overlapping expression of some signature cytokines in different subsets of CD4 $^+$ T cells not only indicate the

plasticity of CD4 $^+$ T cells (Zhu and Paul, 2010), but also suggest the possibility of deletion of a single signature cytokine gene leading to functional differentiation of naïve CD4 $^+$ T cells into Th1 or Tr1 cells under Th2 cell differentiation conditions. In this study, we show that IL-6 and IL-10 gene deficient CD4 $^+$ Th2(IL-6 KO) and Th2(IL-10 KO) cells behave like functional Tr1 and Th1 cells by inhibiting and enhancing DC_{OVA}-induced OVA-specific CD8 $^+$ CTL

responses and antitumor immunity, respectively. We demonstrate that the inhibition of DC_{OVA}-induced OVA-specific CTL responses by CD4⁺ Th2(IL-6 KO) cells is mediated by suppressive IL-10 secretion, consistent with previous reports (Hao et al., 2008; Fiorentino et al., 1991; Steinbrink et al., 1997), since the neutralizing anti-IL-10 Ab treatment completely blocked its inhibitory effect. We also demonstrate that the enhancement of DC_{OVA}-induced OVA-specific CTL responses by CD4⁺ Th2(IL-10 KO) cells is mediated by inflammatory IL-6 secretion since neutralizing anti-IL-6 Ab treatment completely blocked the enhance effect. Our data indicate that potential immune stimulatory and suppressive effect of Th2 cells are balanced by IL-6 and IL-10 cytokines, and shift in this balance is likely lead to potential generation of either immunogenic Th1-like or immune-suppressive Tr1-like cells. Therefore, our study provides the first evidence for another type of CD4⁺ T cell plasticity with converting one type of functional T subset into another by depletion of one single key signature cytokine gene. Since Th2 cells are pathogenic in allergic diseases such as asthma (Gordon et al., 2005), converting Th2 cell lineage commitment with different mechanisms becomes a key target for allergen-specific immunotherapy (Hawrylowicz and O'Garra, 2005; Holgate and Polosa, 2008). Therefore, our study may provide another direction for immunotherapeutic approach to allergic diseases by converting pathogenic Th2 cells into immune suppressive Tr1 cells via blocking IL-6 by neutralizing anti-IL-6 Ab treatment.

5. Conclusions

Taken together, our study suggests that deletion of a single cytokine gene IL-6 and IL-10 makes CD4[±] Th2 cells become effector CD4[±] Tr1- and Th1-like cells, respectively. Our data therefore, not only provide new evidence for another type of CD4⁺ T cell plasticity, but also may have positive impact on the development of a new immunotherapy approach to the treatment of allergic diseases.

Acknowledgments

This study was supported by Canadian Institute of Health Research (MOP 405674). Manjunatha Ankathatti Munegowda was supported by Dean's Scholarship of University of Saskatchewan. We thank Mark Boyd for helping in flowcytometric analysis.

References

- Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., Kuchroo, V.K., 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235–238.
- Boros, D.L., Whitfield, J.R., 1999. Enhanced Th1 and dampened Th2 responses synergize to inhibit acute granulomatous and fibrotic responses in murine schistosomiasis mansonii. *Infect. Immun.* 67, 1187–1193.
- Carter, L.L., Dutton, R.W., 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr. Opin. Immunol.* 8, 336–342.
- Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., Wahl, S.M., 2003. Conversion of peripheral CD4⁺CD25[−] naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J. Exp. Med.* 198, 1875–1886.
- Erdmann, A.A., Jung, U., Foley, J.E., Toda, Y., Fowler, D.H., 2004. Co-stimulated/Tc2 cells abrogate murine marrow graft rejection. *Biol. Blood Marrow Transplant.* 10, 604–613.
- Fernando, G.J., Stewart, T.J., Tindle, R.W., Frazer, I.H., 1998. Th2-type CD4⁺ cells neither enhance nor suppress antitumor CTL activity in a mouse tumor model. *J. Immunol.* 161, 2421–2427.

- Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W., O'Garra, A., 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* 146, 3444–3451.
- Goedegebuure, P.S., Eberlein, T.J., 1995. The role of CD4⁺ tumor-infiltrating lymphocytes in human solid tumors. *Immunol. Res.* 14, 119–131.
- Gordon, J.R., Li, F., Nayyar, A., Xiang, J., Zhang, X., 2005. CD8 alpha⁺, but not CD8 alpha[−], dendritic cells tolerize Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. *J. Immunol.* 175, 1516–1522.
- Hao, S., Yuan, J., Xu, S., Munegowda, M.A., Deng, Y., Gordon, J., Xing, Z., Xiang, J., 2008. Antigen specificity acquisition of adoptive CD4⁺ regulatory T cells via acquired peptide-MHC class I complexes. *J. Immunol.* 181, 2428–2437.
- Hawrylowicz, C.M., O'Garra, A., 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat. Rev. Immunol.* 5, 271–283.
- Holgate, S.T., Polosa, R., 2008. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol.* 8, 218–230.
- Huang, H., Dawicki, W., Zhang, X., Town, J., Gordon, J.R., 2010. Tolerogenic dendritic cells induce CD4⁺CD25hiFoxp3⁺ regulatory T cell differentiation from CD4⁺CD25[−]/loFoxp3[−] effector T cells. *J. Immunol.* 185, 5003–5010.
- Khoruts, A., Miller, S.D., Jenkins, M.K., 1995. Neuroantigen-specific Th2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. *J. Immunol.* 155, 5011–5017.
- Liu, Y., Zhang, P., Li, J., Kulkarni, A.B., Perruche, S., Chen, W., 2008. A critical function for TGF- β signaling in the development of natural CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *Nat. Immunol.* 9, 632–640.
- Lukacs, N.W., Boros, D.L., 1993. Lymphokine regulation of granuloma formation in murine schistosomiasis mansonii. *Clin. Immunol. Immunopathol.* 68, 57–63.
- McKee, A.S., Pearce, E.J., 2004. CD25⁺CD4⁺ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. *J. Immunol.* 173, 1224–1231.
- Ankathatti Munegowda, M., Deng, Y., Mulligan, S.J., Xiang, J., 2011. Th17 and Th17-stimulated CD8(+) T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity. *Cancer Immunol. Immunother.* 60, 1473–1484.
- Oswald, I.P., Caspar, P., Jankovic, D., Wynn, T.A., Pearce, E.J., Sher, A., 1994. IL-12 inhibits Th2 cytokine responses induced by eggs of *Schistosoma mansonii*. *J. Immunol.* 153, 1707–1713.
- Pasare, C., Medzhitov, R., 2003. Toll pathway-dependent blockade of CD4⁺CD25⁺ T cell-mediated suppression by dendritic cells. *Science* 299, 1033–1036.
- Porgador, A., Yewdell, J.W., Deng, Y., Binnink, J.R., Germain, R.N., 1997. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* 6, 715–726.
- Roncarolo, M.G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., Levings, M.K., 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol. Rev.* 212, 28–50.
- Skapenko, A., Kalden, J.R., Lipsky, P.E., Schulze-Koops, H., 2005. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25⁺CD4⁺ regulatory T cells from CD25[−]CD4⁺ precursors. *J. Immunol.* 175, 6107–6116.
- Steinbrink, K., Wolff, M., Jonuleit, H., Knop, J., Enk, A.H., 1997. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* 159, 4772–4780.
- Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., Glimcher, L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100, 655–669.
- Takeda, K., Kaisho, T., Yoshida, N., Takeda, J., Kishimoto, T., Akira, S., 1998. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J. Immunol.* 161, 4652–4660.
- Tone, Y., Furuuchi, K., Kojima, Y., Tykocinski, M.L., Greene, M.I., Tone, M., 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat. Immunol.* 9, 194–202.
- Umehappa, C.S., Huang, H., Xie, Y., Wei, Y., Mulligan, S.J., Deng, Y., Xiang, J., 2009. CD4⁺ Th-APC with acquired peptide/MHC class I and II complexes stimulate type 1 helper CD4⁺ and central memory CD8⁺ T cell responses. *J. Immunol.* 182, 193–206.
- Waaga, A.M., Gasser, M., Kist-van Holthe, J.E., Najafian, N., Muller, A., Vella, J.P., Womer, K.L., Chandraker, A., Khoury, S.J., Sayegh, M.H., 2001. Regulatory functions of self-restricted MHC class II allopeptide-specific Th2 clones in vivo. *J. Clin. Invest.* 107, 909–916.
- Wilson, C.B., Rowell, E., Sekimata, M., 2009. Epigenetic control of T-helper-cell differentiation. *Nat. Rev. Immunol.* 9, 91–105.
- Xiang, J., Huang, H., Liu, Y., 2005. A new dynamic model of CD8⁺ T effector cell responses via CD4⁺ T helper-antigen-presenting cells. *J. Immunol.* 174, 7497–7505.
- Zheng, W., Flavell, R.A., 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89, 587–596.
- Zhu, J., Paul, W.E., 2010. Heterogeneity and plasticity of T helper cells. *Cell Res.* 20, 4–12.