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IL-12 inhibits the TGF-β-dependent T cell developmental programs and skews the TGF-β-induced differentiation into a Th1-like direction

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ABSTRACT

The development and differentiation of T helper (Th) cell subsets is a highly plastic process which is strictly regulated by cytokines. Here we show that the transforming growth factor β (TGF-β)-dependent differentiation programs are negatively regulated by interleukin-12 (IL-12). The development of TGF-β-induced regulatory T cells (iTregs) or TGF-β/IL-6 activated Th17 cells from purified mouse $CD4^+CD25^-$ T cells, stimulated with monoclonal antibody anti-CD3, was abrogated in the presence of IL-12 and a different developmental program was established. On the molecular level, IL-12 inhibited the expression of the lineage specific transcription factors Foxp3 and RORγt in developing Tregs and Th17 cells, respectively. Moreover, IL-12 was able to alter the development of iTregs and Th17 cells even when added to the differentiating cells after 48 h of the culture. The cells activated in the presence of TGF-β and IL-12 had an increased expression of the Th1 transcription factor T-bet, produced Th1 cytokines interferon γ and IL-2 and expressed IL-18 receptor and C-C chemokine receptor type 5 which are the phenotypic markers characteristic for Th1 cells. Furthermore, the cells activated in the presence of both TGF-β and IL-12, and not of TGF-β only, stimulated macrophages to produce nitric oxide. Altogether, these results indicate that IL-12 is a superior cytokine that has the ability to skew the already ongoing TGF-β-dependent iTreg or Th17 developmental program into Th1-like direction.

Introduction

The development and differentiation of naive $CD4^+$ cells into several T helper (Th) cell subsets or regulatory T cells (Tregs) is strictly regulated by cytokines. In this respect, interleukin-12 (IL-12) is essential for the development of Th1 proinflammatory cells (Manetti et al. 1993; O'Shea and Paul 2002) and IL-4 is necessary for the generation of Th2 cell population (Glimcher and Murphy 2000; Swain et al. 1990). Another cytokine, transforming growth factor β (TGF-β), plays a crucial role in the development of Tregs, but the presence of some other cytokines together with TGF-β may lead to the differentiation of functionally distinct cell types. Th17 proinflammatory cells were shown to develop in the presence of TGF-β in combination with IL-6 (Bettelli et al. 2006). Similarly, the proinflammatory Th9 cells arise after stimulation of T cells in the presence of TGF-β and IL-4 (Dardalhon et al. 2008).

Individual Th cell subtypes are characterized by the expression of specific transcription factors and by the production of various cytokines. Th1 cells express the transcription factor T-bet and secrete interferon γ (IFN-γ) and IL-2 (Szabo et al. 2000). Both T-bet and IFN-γ suppress the development of Th2 cells (Djuretic et al. 2007). The transcription factor GATA-3 is typical for Th2 cells (Lee et al. 2000; Zheng and Flavell 1997). It activates the expression of IL-4, IL-5 and IL-13 and suppresses the transcription of IFN-γ which, together with IL-4, antagonizes Th1 cell differentiation (Ansel et al. 2004; Mullen et al. 2001; Smeltz et al. 2002; Zhang et al. 1997). Tregs are characterized by the presence of forkhead box p3 (Foxp3), a transcription factor induced by TGF-β (Chen et al. 2003; Hori et al. 2003). The proinflammatory Th17 cells express the transcription factor retinoic acid receptor-related orphan receptor γt (RORγt) that is also induced by TGF-β. However, Th17 cell development requires the presence of IL-6 which leads to the suppression of simultaneous Foxp3 expression (Ivanov et al. 2006; Mangan et al. 2006; Yang et al. 2008). TGF-β, on the other hand, induces the expression of Foxp3 which is subsequently able to inhibit Th17 cell

Abbreviations: IL-12, interleukin-12; TGF-β, transforming growth factor β; Th, helper T cell; NO, nitric oxide; IFN-γ, interferon γ; PE, phycoerythrin; CFSE, carboxyfluorescein succinimidyl ester; ConA, concanavalin A; LPS, lipopolysacharide; PBS, phosphate buffered saline; CCR5, C-C chemokine receptor type 5; Tregs, regulatory T cells; nTregs, naturally occuring regulatory T cells; iTregs, induced regulatory T cells; RORγt, retinoic acid receptor-related orphan receptor γt; Foxp3, forkhead box p3; mAb, monoclonal antibody.

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differentiation by antagonizing the function of RORγt (Zhou et al. 2008).

It has been demonstrated that TGF-β-induced differentiation of Tregs can be abrogated by various cytokines, including IL-4, IL-12 or IFN-γ (Mantel et al. 2007; Prochazkova et al. 2009; Wei et al. 2007). Similarly, it has been shown that the differentiation of Th17 cells is prevented by IFN-γ and IL-4, the product of Th1 and Th2 cells, respectively (Lexberg et al. 2008). IL-12 determines Th1 cell differentiation, but its role in the commitment of other T-cell lineages remains controversial. Taken together, cytokines are the major determining factors of T cell differentiation and their combinations might direct the development in yet undescribed ways.

So far, developmental plasticity of Th cells has been studied in various models, but many aspects of T-cell differentiation remain unclear (Dardalhon et al. 2008). In the present study, we tested the role of IL-12 in TGF-β-dependent differentiation programs of $CD4^+CD25^-$ cells and in developmental plasticity of inhibitory iTregs and proinflammatory Th17 cells. We show that IL-12 suppresses the expression of the lineage specific transcriptional factors Foxp3 and RORγt and thus prevents the differentiation of iTregs and Th17 cells. Moreover, we demonstrate that these TGF-β-dependent developmental programs are skewed in the presence of IL-12 into a Th1-like direction.

Materials and methods

Mice

Mice of both sexes of the inbred strain BALB/c at the age 8–10 weeks obtained from the breeding unit of the Institute of Molecular Genetics, Prague were used in the experiments. The use of the animals was approved by the Local Ethical Committee of the Institute of Molecular Genetics.

Separation of T cell subpopulations

$CD4^+CD25^-$ and $CD4^+CD25^+$ cell subpopulations were isolated from mouse spleen single-cell suspensions using $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and the AutoMACS magnetic separation system (Miltenyi Biotec). In brief, spleen cells were first magnetically labeled with a cocktail of biotin-conjugated antibodies and Anti-Biotin MicroBeads to exclude non-CD4 cells. Consequently, the cells were stained with anti-CD25-phycoerythrin (PE) antibody. For the isolation of $CD4^+CD25^+$ cells, the anti-CD25 PE-labeled cells in the CD4 fraction were magnetically labeled with anti-PE MicroBeads and two distinct populations of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were isolated. Flow cytometry analysis of the sorted cells showed that over 96% of cells in the $CD4^+CD25^-$ population were $CD25^-$ and more than 99% of cells were $Foxp3^-$ and $ROR\gamma t^-$. In addition, more than 85% of the $CD4^+CD25^-$ cells express CD62L, a marker of naïve T cells (data not shown). The purified $CD4^+CD25^+$ cells were used as naturally occuring Tregs (nTregs) control in some experiments.

Cell stimulation and induction of iTreg and Th17 cells

Purified $CD4^+CD25^-$ cells (0.5×10^6/ml) were cultured in a volume of 1 ml of RPMI 1640 medium (Sigma Chemical Corp. St. Louis, MO) containing 10% heat-inactivated FCS (Sigma), antibiotics (100 U/ml of penicillin, 100 mg/ml of streptomycin), 10 mM HEPES buffer and 5×10^{-5} M 2-ME (complete RPMI 1640 medium) in 48-well tissue culture plates (Corning Corp., Corning, NY) alone or stimulated with 1 μg/ml of immobilized mAb anti-CD3 (clone KT3; Tomonari 1988) and in the presence or absence of 3 ng/ml of TGF-β (PeproTech, Rocky Hill, NJ) and 10 ng/ml of IL-12 (PeproTech) or TGF-β (3 ng/ml) and IL-6 (10 ng/ml, PeproTech). After 96-h incubation period, the cells were harvested and the expression of Foxp3 and RORγt was analyzed by qPCR or the population of $CD4^+Foxp3^+$ and $CD4^+ROR\gamma t^+$ cells was determined by FACS analysis. As control cells, the Th1, Th2 and Th17 lineages were prepared. The purified $CD4^+CD25^-$ cells were stimulated with mAb anti-CD3 (1 μg/ml) and IL-2 (10 ng/ml, PeproTech) in the presence of IL-12 (10 ng/ml) plus mAb anti-IL-4 (20 μg/ml, eBioscience) for the differentiation of Th1 cells, in the presence of IL-4 (10 ng/ml, PeproTech) plus mAb anti-IL-12 (20 μg/ml, eBioscience) for Th2 cells or in the presence of TGF-β (3 ng/ml) plus IL-6 (10 ng/ml) for Th17 cells. The cells were harvested after 96 h and used as controls for qPCR or were restimulated with concanavalin A (ConA, Sigma) for 24 h and the cells and supernatants were used as controls for qPCR and ELISA, respectively.

Intracellular fluorescence staining and proliferation tracking

The cultured cells were harvested and washed with PBS containing 0.5% BSA. Before intracellular staining, the cells were incubated for 20 min on ice with FITC-labeled mAb CD4 (clone GK1.5; BD Bioscience Pharmingen, San Jose, CA), allophycocyanine-labeled anti-CD25 (clone PC 61.5; eBioscience) and Live/Dead Fixable Violet Dead Cell Stain Kit (Molecular Probes, Eugene, OR) for staining of dead cells. Cells were washed in PBS/0.5% BSA, fixed and permeabilized using Foxp3 buffer staining set (eBioscience) according to the manufacturer's instructions before staining for intracellular Foxp3 and RORγt expression using PE-Cy5-conjugated anti-mouse/rat FoxP3 antibody (clone FJK-16s, eBioscience) and PE-conjugated anti-mouse/human RORγt antibody (clone AFKJS-9; eBioscience) for 30 min. Data was collected using LSRII cytometer (BD Bioscience) and analyzed using FlowJo software (Treestar, Ashland, OR).

In some experiments, the reactive cells were labeled before culture by 5-min incubation at 37 °C with 5 μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes). The proliferation of labeled cells was evaluated by gradual CFSE dilution in dividing T cells by flow cytometry.

Table 1
Sequence of primers used for qPCR.

Gene	5′–3′ left primer sequence	5′–3′ right primer sequence
GAPDH	AGA ACA TCA TCC CTG CAT CC	ACA TTG GGG GTA GGA ACA C
Foxp3	GCT ACG ATG CAG CAA GAG C	AGA AGC TGG GAG CTA TGC AG
T-bet	TTC AAC CAG CAC CAG ACA GA	ACA TCC TGT AAT GGC TTG TGG
GATA-3	TTA TCA AGC CCA AGC GAA G	TGG TGG TGG TCT GAC AGT TC
RORγt	TCC CAC ATC TCC CAC ATT G	ACC TCT TTT CAC GGG AGG A
IL-2	TTC AAT TCT GTG GCC TGC TT	GCT GTT GAT GGA CCT ACA GGA
IFN-γ	TTC AAG ACT TCA AAG AGT CTG AGG	ATC TGG AGG AAC TGG CAA AA
IL-4	TCT GTG GTG TTC TTC GTT GC	GAG AGA TCA TCG GCA TTT TGA
IL-5	ACA TTG ACC GCC AAA AAG AG	ATC CAG GAA CTG CCT CGT C
IL-18R	TGG AAT TCT GGC CAG TTG A	TCC AAT TGC GAC GAT CAT T
CCR5	GAG ACA TCC GTT CCC CCT AC	GTC GGA ACT GAC CCT TGA AA