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TGF-β is necessary for induction of IL-23R and Th17 differentiation by IL-6 and IL-23

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ABSTRACT

TGF-β and IL-6 induce Th17 differentiation, and IL-23 is required for expansion and maintenance of Th17 cells. Recently, it was shown that IL-6 up-regulates IL-23R mRNA in naive CD4$^+$ T cells and therefore IL-6 and IL-23 synergistically promote Th17 differentiation. However, the molecular mechanism whereby IL-6 and IL-23 induce Th17 differentiation and the relevance to TGF-β remain unknown. Here, we found that IL-6 up-regulated IL-23R mRNA expression, and IL-6 and IL-23 synergistically augmented its protein expression. The combination induced Th17 differentiation, and TGF-β1 further enhanced it. IL-6 augmented endogenous TGF-β1 mRNA expression, whereas the amount of TGF-β produced was not enough to induce Th17 differentiation by IL-6 alone. However, unexpectedly, the up-regulation of IL-23R and induction of Th17 differentiation by IL-6 and IL-23 were almost completely inhibited by anti-TGF-β. These results suggest that the induction of IL-23R and Th17 differentiation by IL-6 and IL-23 is mediated through endogenously produced TGF-β.

Introduction

A novel T helper cell subset producing IL-17, called Th17, that mediates the inflammation associated with tissue-specific autoimmune diseases including experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) has been elucidated [1–3]. Aggarwal et al. demonstrated that IL-23 elicits production of the proinflammatory cytokine IL-17 from CD4$^+$ T cells of the effector and memory, but not naive, phenotype [4]. Langrish et al. also demonstrated that adoptive transfer of autoantigen-specific Th17 cells generated in vivo by IL-23 but not Th1 cells by IL-12, induces EAE as well as CIA [5]. Thereafter, Harrington et al. [6] and Park et al. [7] demonstrated that IL-23 can drive in vitro Th17 differentiation from naive CD4$^+$ T cells when IFN-γ and IL-4 are neutralized by blocking Abs or genetic deficiency. Thus, IL-23 appeared to be required for Th17-mediated immunopathology. However, three new reports indicated that IL-23 is not essential for development of Th17 but that instead Th17 differentiation is driven by the combination of TGF-β and IL-6 [8–10]. TGF-β is an important cytokine that promotes the differentiation of anti-inflammatory Foxp3$^+$ regulatory T (Treg) cells [11]. Bettelli et al. demonstrated that IL-6 is capable of inhibiting TGF-β-dependent Foxp3$^+$ Treg cell induction and simultaneously TGF-β plus IL-6 promotes Th17 cell differentiation [9]. Mangan et al. also showed that in Th17-polarizing conditions induced by TGF-β, TGF-β induces IL-23R expression and confers IL-23 responsiveness and that IL-23 subsequently serves as a survival factor for committed Th17 [10]. More recently, the transcription factors RORγt and STAT3 were shown to be critically important for the development of Th17 cells [12–15]. Taken together, these recent studies indicate that TGF-β and IL-6 plays a critical role in the differentiation of naive CD4$^+$ T cells to Th17 cells, and that IL-23 is important for amplifying and/or stabilizing the Th17 phenotype.

Recently, it was further demonstrated that IL-6 functions to up-regulate IL-23R mRNA in naive CD4$^+$ T cells and that IL-23 synergizes with IL-6 in promoting Th17 differentiation [15]. However, the molecular mechanism whereby IL-6 and IL-23 induce Th17 differentiation and the relevance to TGF-β remain unknown. In the present study, we found that IL-6 up-regulates IL-23R mRNA expression, whereas IL-23 is necessary to induce IL-23R at protein level in synergy with IL-6. Moreover, IL-6 augmented TGF-β mRNA expression in naive CD4$^+$ T cells, but IL-6 alone was not enough to induce Th17 differentiation. Of note is that the induction of IL-23R and Th17 differentiation by IL-6 and IL-23 was highly dependent on TGF-β, which was produced endogenously by IL-6. Thus, the combination of IL-6 and IL-23 may play an important role for the induction of Th17 differentiation under certain circumstances where bioactive TGF-β production is limited.

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Materials and methods

Cell culture and mice. Naive CD4$^+$ T cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 μM 2-ME. BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Mice transgenic for αβ TCR recognizing $OVA_{323-339}$ (DO11.10, BALB/c background) were provided by Dr. T. Yoshimoto (Hyogo College of Medicine, Hyogo, Japan). STAT1-deficient mice of 129/Sv background and wild-type 129/Sv mice were purchased from Taconic Farms, Inc. (Germantown, NY). T-bet-deficient mice with BALB/c background were purchased from Jackson Laboratory (Bar Harbor, ME). Mice lacking STAT3 specifically in T cells (Lck-Cre/$STAT3^{flox/flox}$) were generated by mating $STAT3^{flox/flox}$ mice, in which the STAT3 gene is flanked by two loxP sites, and Lck-Cre transgenic mice (purchased from Center for Animal Resources and Development, Kumamoto University, Kumamoto, Japan), in which the Cre recombinase transgene is regulated by T cell specific Lck promoter. $STAT3^{flox/flox}$ mice were used as their control wild-type mice. All animal experiments were performed in accordance with our Institutional Guidelines.

Reagents. Anti-actin was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-CD3 (145-2C11), anti-IL-4 (11B11) and anti-IFN-γ (XMG1.2) were from American Type Culture Collection (ATCC, Manassas, VA). FITC-anti-CD4 (GK1.5) and anti-CD28 (37.51) were from eBioscience (San Diego, CA). Anti-CD25-PE (PC61), APC-anti-CD44 (1M7), anti-IL-17 (TC11-18H10.1), biotin-anti-IL-17 (TC11-8H4.1) and PE-anti-IL-17 (TC11-18H10.1) were from BD Biosciences (Franklin Lakes, NJ). Mouse recombinant (r)IL-17, mouse rIL-23, human rIL-6, anti-IL-23R and anti-TGF-β (1D11) were from R&D Systems (Minneapolis, MN). Human rTGF-β1 was from Peprotech Inc. (Rocky Hill, NJ). Human rIL-2 was kindly provided by Shionogi & Co. Ltd. (Osaka, Japan). Mouse recombinant soluble (s)IL-6R/IL-6 was prepared as a fusion protein by flexibly linking sIL-6Rα to IL-6 similarly as described before [16,17].

Preparation of naive CD4$^+$ T cells. Primary T cells were purified by passing spleen cells depleted of erythrocytes through nylon wool. The flow-through fraction was incubated with biotin-conjugated anti-CD8α anti-B220, anti-Mac-1, anti-Ter-119, and anti-DX5, followed by incubation with anti-biotin magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and passed through a magnetic cell sorting column using AutoMACS (Miltenyi Biotec), and the negative fraction was collected (CD4$^+$ T cells > 95%). These purified T cells were then incubated with anti-CD62L magnetic beads (Miltenyi Biotec), and the positive fraction was collected as purified naive CD4$^+$CD62L$^+$ T cells (CD62L$^+$ cells > 99%). To obtain a further purified population of naive CD4$^+$CD25$^-$CD44^{low}CD62L$^+$ T cells, cell sorting was performed on a FACSVantage (BD Biosciences) after staining of MACS-sorted CD4$^+$CD62L$^+$ T cells as described above with FITC-anti-CD4, PE-anti-CD25 and APC-anti-CD44 (> 99% purity).

Primary stimulation assay. Naive CD4$^+$ T cells (2×10^5 cells/ml) were activated with plate-coated anti-CD3 (2 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence of anti-IFN-γ and anti-IL-4 (10 μg/ml each). After 72 h or the indicated time, culture supernatants were collected and assayed for IL-17 production by ELISA according to the manufacturer's instruction (BD Biosciences). Acti-

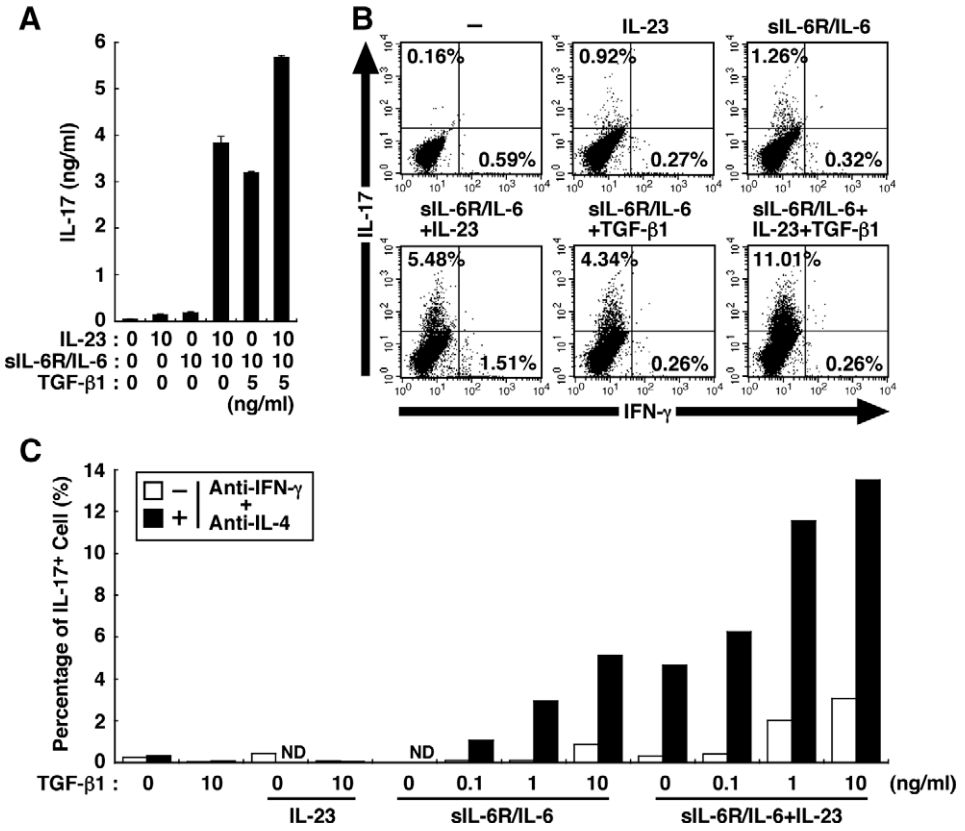

Fig. 1. IL-6 and IL-23 induces Th17 differentiation and TGF-β enhances it. (A) Naive CD4$^+$ T cells were activated with plate-coated anti-CD3 and anti-CD28 in the presence of anti-IFN-γ and anti-IL-4. Various combinations of sIL-6R/IL-6 (10 ng/ml), IL-23 (10 ng/ml), and TGF-β1 (5 ng/ml) were also included. These activated cells were expanded with IL-2 on day 3, harvested on day 5, and restimulated with plate-coated anti-CD3 for 24 h. Culture supernatant was collected and assayed for IL-17 production in triplicate by ELISA. Data are shown as the mean ± SD. (B) These activated and harvested cells on day 5 as described above, were restimulated with PMA and ionomycin for 6 h, and analyzed by intracellular staining for IL-17 and IFN-γ. (C) Naive CD4$^+$ T cells were activated with plate-coated anti-CD3 and anti-CD28 in the presence or absence of anti-IFN-γ and anti-IL-4. Various combinations of sIL-6R/IL-6 (10 ng/ml), IL-23 (10 ng/ml), and TGF-β1 (0.1, 1 and 10 ng/ml) were also included. These activated cells were expanded, restimulated with PMA and ionomycin, and analyzed by intracellular staining for IL-17 and IFN-γ. Similar results were obtained in at least two independent experiments.

vated cells were also harvested and subjected to RT-PCR and Western blotting.

Th17 differentiation assay. Naive $CD4^+$ T cells (2×10^5 cells/ml) were activated with plate-coated anti-CD3 (2 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence of anti-IFN-γ and anti-IL-4 (10 μg/ml each). DO11.10 TCR-transgenic naive $CD4^+$ T cells (2×10^5 cells/ml) were activated with $OVA_{323-339}$ peptide and irradiated splenic DC (4×10^4 cells/ml) as APC in the presence of anti-IFN-γ and anti-IL-4. Splenic DCs were positively purified with anti-CD11c (N418) magnetic beads and AutoMACS from adherent cells of collagenase-digested spleen ($CD11c^+I\text{-}A^+$ DCs > 90%). On day 3, these activated cells were expanded with equal volumes of IL-2 (40 U/ml)-containing complete medium. On day 5, cells were collected, washed and restimulated at 1×10^6 cell/ml with plate-coated anti-CD3 (2 μg/ml). After 24 h, culture supernatants were collected and assayed for IL-17 production by ELISA. For intracellular staining with PE-anti-IL-17 and FITC-anti-IFN-γ, washed cells were restimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 6 h and monensin (Sigma) was added for last 5 h. Resultant cells were analyzed for IL-17- or IFN-γ-expressing population by FACSCalibur (BD Biosciences).

RT-PCR analysis. Total RNA was extracted using a guanidine thiocyanate procedure, cDNA was prepared using oligo(dT) primer and SuperScript RT (Invitrogen Corp. Carlsbad, CA), and RT-PCR was then performed using Taq DNA polymerase as described [18]. Primers used for hypoxanthine phosphoribosyl transferase (HPRT) were described [19]. Following primers were also used; IL-23R sense primer, 5′-TCAATTCCCTAGGCATGGAG-3′; IL-23R antisense primer, 5′-GTTC GTGGGATGATTTTGCT-3′; TGF-β1 sense primer, 5′-TGACGTCACTGG AGTTGTACGG-3′; TGF-β1 antisense primer, 5'-GGTTCATGTCATGG ATGGTGC-3′. The amplified products were size fractionated by electrophoresis on an agarose gel, followed by ethidium bromide staining for UN-assisted visualization. The intensity of each band was densitometrically measured, and relative intensity was calculated after normalization by the intensity for HPRT.

Statistical analysis. Statistical analysis was performed by Student's *t-test. A p value of less than 0.05 was considered to indicate statistical significance.*

Results

IL-6 and IL-23 induces Th17 differentiation and TGF-β enhances it

It was previously demonstrated that IL-6 up-regulates IL-23R mRNA expression and therefore IL-6 and IL-23 synergistically induce Th17 differentiation [15]. To investigate the molecular mechanism whereby IL-6 and IL-23 induce Th17 differentiation, we first confirmed the effect of IL-6 and IL-23 on induction of Th17 differentiation. Naive $CD4^+$ T cells ($CD4^+CD25^-CD44^{low}CD62L^+$) were purified by FACS sorting from spleen cells of BALB/c mice and activated with plate-coated anti-CD3/anti-CD28 in the presence of sIL-6R/IL-6, IL-23 and/or TGF-β1. In most of the present experiments, we used a recombinant fusion protein of mouse sIL-6Ra and mouse IL-6 (sIL-6R/IL-6) in place of IL-6, since this fusion protein is capable of inducing signals that are similar to but stronger than IL-6 through gp130 [16,20]. On day 3 these activated cells were expanded with IL-2, and restimulated with plate-coated anti-CD3 on day 5 for 24 h. Culture supernatants were collected and assayed for IL-17 production by ELISA. Consistent with the previous report [15], the combination of sIL-6R/IL-6 and IL-23 efficiently induced IL-17 production, while each cytokine alone failed to do so (Fig. 1A). Similar results were obtained, when human IL-6 was used in place of sIL-6R/IL-6 (data not shown). Intracellular staining for IL-17 and IFN-g after restimulation with PMA and ionomycin also revealed the induction of IL-17-expressing population by the combination of sIL-6R/IL-6 and IL-23 (Fig. 1B). The effect of sIL-6R/IL-6

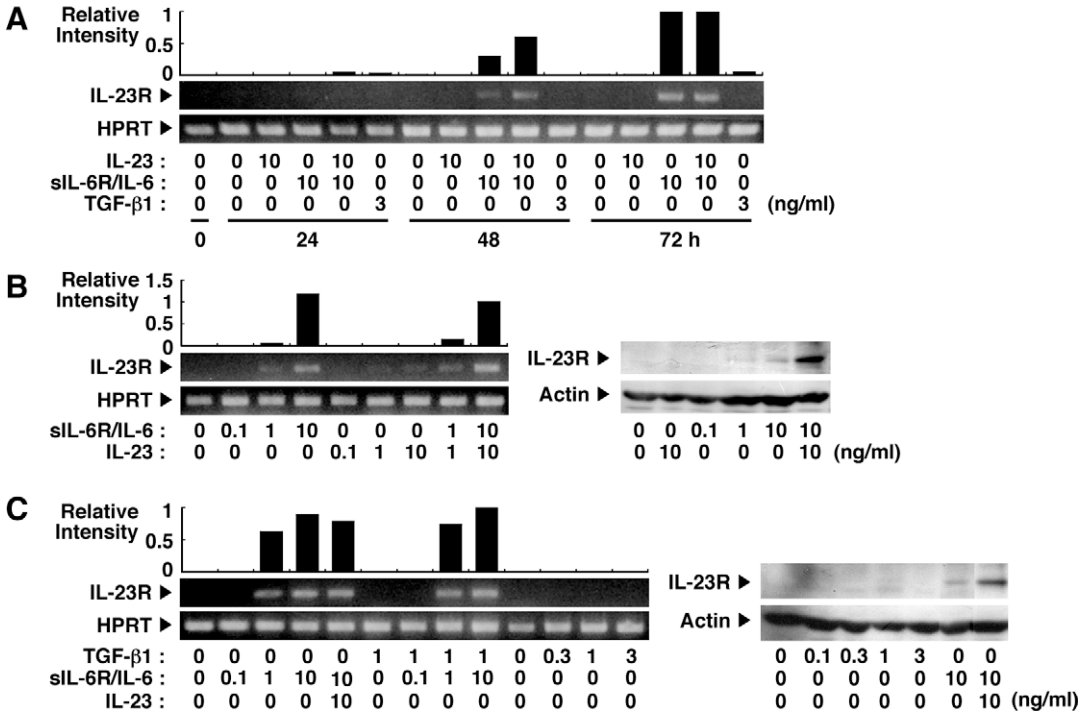

Fig. 2. IL-6 enhances IL-23R mRNA expression and IL-6 and IL-23 synergistically augment its protein expression. Naive $CD4^+$ T cells were activated with plate-coated anti-CD3 and anti-CD28 in the presence of anti-IFN-γ and anti-IL-4. Various concentrations of sIL-6R/IL-6, IL-23 and/or TGF-β1 were also included. After indicated time (A) or 72 h unless indicated (B and C), these activated cells were harvested, and total RNA was prepared to analyze for mRNA expression of IL-23R and HPRT as control by RT-PCR. The intensity of each band was densitometrically measured, and relative intensity was calculated after normalization by the intensity for HPRT. Total cell lysate was also prepared and subjected to Western blotting with anti-IL-23R and anti-actin as control (B and C). Similar results were obtained in three independent experiments.

and IL-23 appears to be comparable to that induced by the combination of TGF-β1 and sIL-6R/IL-6 (Fig. 1B). The addition of TGF-β1 further enhanced the effect of IL-6 and IL-23 (Fig. 1A and B), and the dose-dependent augmentation of IL-17-expressing population by TGF-β1 was also observed (Fig. 1C). Similar induction of Th17 differentiation by sIL-6R/IL-6 and IL-23 was observed when DO11.10 TCR-transgenic naive $CD4^+$ T cells were activated with OVA peptide-pulsed splenic DC as APC (data not shown). Thus, IL-6 and IL-23 synergistically induces Th17 differentiation, and TGF-β1 further enhances it.

IL-6 induces IL-23R mRNA expression and IL-6 and IL-23 synergistically augment its protein expression

We next examined the effect of IL-6 and IL-23 on IL-23R expression. Naive $CD4^+$ T cells were activated with plate-coated anti-CD3/anti-CD28, sIL-6R/IL-6 and/or IL-23, and analyzed for IL-23R expression by RT-PCR and Western blotting. Consistent with the previous report [15], we observed significant up-regulation of IL-23R expression at mRNA level by sIL-6R/IL-6 in a time- and dose-dependent manner (Fig. 2A and B). Moreover, the sIL-6R/IL-6-induced IL-23R up-regulation was greatly enhanced by IL-23 not only at mRNA level but also at protein level. So far, mAb specific to IL-23R and applicable to FACS analysis has not been commercially available. Therefore, we cannot currently determine the cell surface expression of IL-23R by FACS. Since TGF-β was also reported to up-regulate IL-23R expression [10], we next compared the ability of sIL-6R/IL-6 and TGF-β1 to up-regulate IL-23R expression (Fig. 2A and C). TGF-β1-induced IL-23R up-regulation was hardly observed until 72 h after the stimulation, whereas sIL-6R/IL-6 markedly induced it. In the previous paper [10], TGF-β-induced IL-23R up-regulation was determined only on day 6 after the stimulation. These results suggest that IL-6 rapidly and efficiently induces IL-23R mRNA up-regulation in naive $CD4^+$ T cells, and that the combination of IL-6 and IL-23 synergistically augments IL-23R expression at protein level.

IL-23R up-regulation and Th17 differentiation induced by IL-6 and IL-23 are mediated through endogenously produced TGF-β

It was recently reported that STAT3 activation directly elevates TGF-β promoter activity [21]. In addition, TGF-β was demonstrated to be required to drive naive $CD4^+$ T cells to Th17 cells [8–10]. Therefore, we next explored a role for TGF-β in the IL-6 and IL-23-mediated induction of Th17 differentiation. We first examined whether IL-6 augments the endogenous TGF-β1 mRNA expression

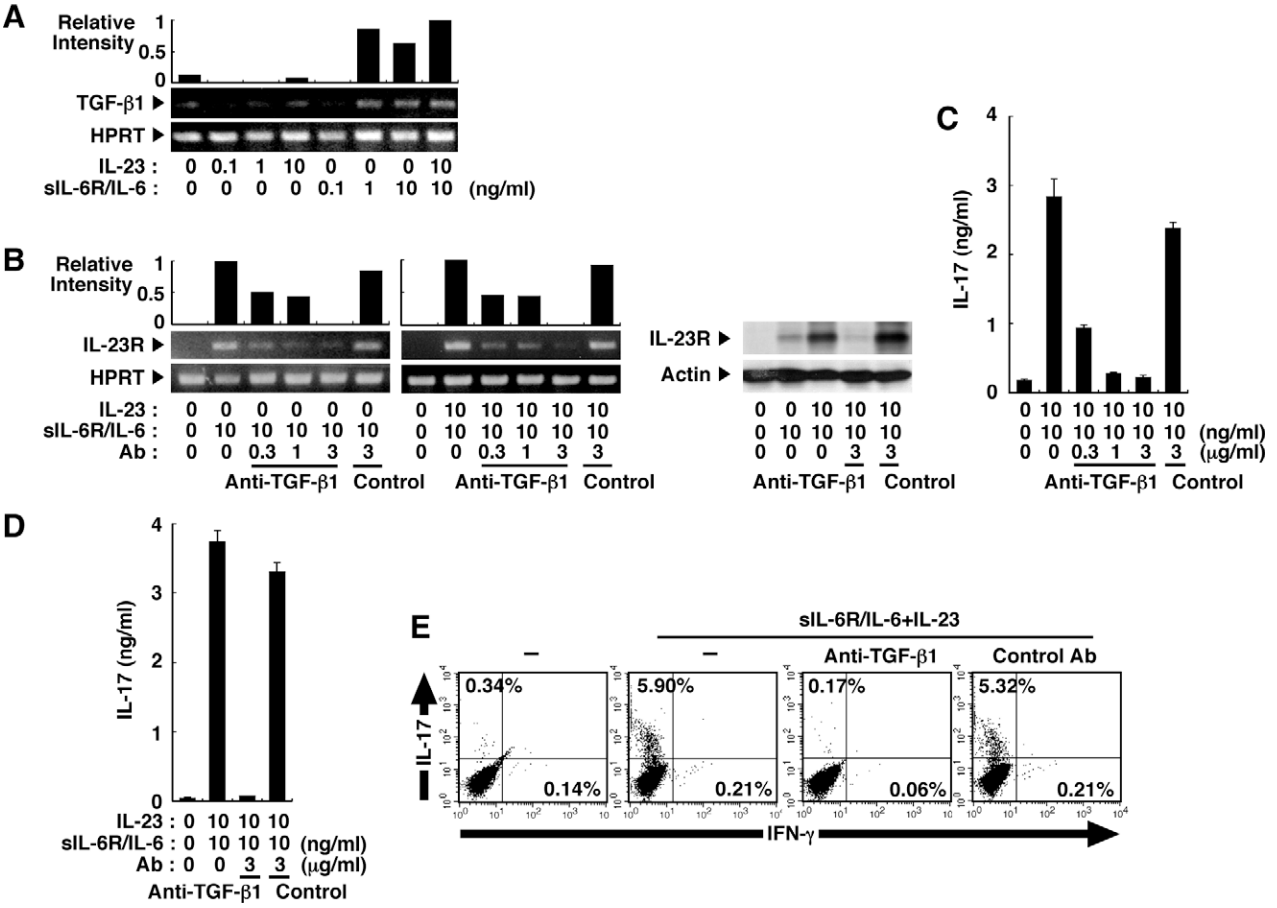

Fig. 3. IL-23R up-regulation and Th17 differentiation induced by IL-6 and IL-23 are mediated through endogenously produced TGF-β. (A) Naive $CD4^+$ T cells were activated with plate-coated anti-CD3/anti-CD28, sIL-6R/IL-6 and/or IL-23 in the presence of anti-IFN-γ and anti-IL-4. After 72 h, these activated cells were harvested to analyze for TGF-β1 mRNA expression by RT-PCR. The intensity of each band was densitometrically measured, and relative intensity was calculated after normalization by the intensity for HPRT. (B) Naive $CD4^+$ T cells were also activated with plate-coated anti-CD3/anti-CD28 and sIL-6R/IL-6 or sIL-6R/IL-6 plus IL-23 in the presence of anti-IFN-γ, anti-IL-4, and anti-TGF-β or control Ab. After 72 h, activated cells were harvested and subjected to RT-PCR analysis as described above and Western blotting for IL-23R expression at mRNA and protein levels, respectively. (C) Culture supernatants were also collected and assayed for IL-17 production in triplicate by ELISA. Data are shown as the mean ± SD. (D and E) Naive $CD4^+$ T cells were activated with plate-coated anti-CD3/anti-CD28, sIL-6R/IL-6 (10 ng/ml) and/or IL-23 (10 ng/ml) in the presence of anti-IFN-γ, anti-IL-4, and anti-TGF-β (3 mg/ml) or control Ab (3 mg/ml), expanded, and restimulated with plate-coated anti-CD3 and PMA/ionomycin for ELISA and intracellular staining, respectively. Similar results were obtained in at least two independent experiments.

by RT-PCR. Consistent with a previous report [21], sIL-6R/IL-6 slightly but repeatedly augmented TGF-β1 mRNA expression (Fig. 3A). However, we have not succeeded in the detection of bioactive production of TGF-β1 by its specific ELISA and a bioassay using TGF-β-responsive MFB-F11 cells [22] (data not shown). Therefore, we next examined the effect of a neutralizing mAb against TGF-β. The anti-TGF-β, but not control Ab, almost completely inhibited IL-23R up-regulation induced by sIL-6R/IL-6 alone and by the combination of sIL-6R/IL-6 and IL-23 at mRNA and protein levels in a dose-dependent manner (Fig. 3B). Moreover, anti-TGF-β1 almost completely inhibited sIL-6R/IL-6- and IL-23-induced primary IL-17 production (Fig. 3C) and Th17 differentiation (Fig. 3D and E) as well. Further analyses using naive $CD4^+$ T cells obtained from deficient mice for STAT1, STAT3 and T-bet revealed that STAT3 but not STAT1 and T-bet is important for IL-23R up-regulation and Th17 differentiation induced by IL-6 and IL-23 (data not shown). These results suggest that IL-23R up-regulation, primary IL-17 production and Th17 differentiation induced by IL-6 and IL-23 are highly dependent on endogenously produced TGF-β.

Discussion

It was previously shown that up-regulation of IL-23R expression by TGF-β was observed 6 days after the stimulation [10], whereas TGF-β alone failed to efficiently induce IL-23R up-regulation during the first 72 h after the stimulation (Fig. 2A). In contrast, IL-6 rapidly and efficiently up-regulated IL-23R at mRNA level and the combination of IL-6 and IL-23 further augmented the expression, especially, at protein level (Fig. 2). Moreover, IL-6 augmented TGF-β1 mRNA expression in naive $CD4^+$ T cells (Fig. 3A), which is consistent with recent reports showing that STAT3 activation directly enhances TGF-β promoter activity [21] and also that T cell is a cellular source of TGF-β important for Th17 differentiation [23]. TGF-β is secreted in a latent or inactive form that requires proteolytic, conformational, and/or acidic conditions to remove the latency-associated peptide, liberating the biologically active mature TGF-β recognized by transmembrane serine–threonine kinase TGF-β receptors [24]. Therefore, the regulation of TGF-β activity is very complex and multifactorial. Currently, there are mainly two methods to sensitively and specifically detect a bioactive form of TGF-β; one is an ELISA using mAbs specific for bioactive form and the other is a bioassay using TGF-β-responsive cells including MFB-F11 [22]. We also tried to detect the production of bioactive form of TGF-β using these methods, but we have not succeeded in it. However, neutralizing Ab against TGF-β almost completely blocked the effect of IL-6 and IL-23 (Fig. 3B–E), suggesting the production of traces of bioactive form of TGF-β in the culture is necessary. Therefore, the induction of Th17 differentiation could be attributed to the cooperative effect of not only IL-6 and IL-23 but also endogenously produced TGF-β.

Although IL-23R is not expressed on naive $CD4^+$ T cells [25], IL-6 thus rapidly and efficiently up-regulates IL-23R expression on naive $CD4^+$ T cells through STAT3 and TGF-β in synergy with IL-23, resulting in augmentation of Th17 differentiation. This scenario has much analogy to that of IFN-γ/IL-12Rβ2/IL-12 [26]. That is, IFN-γ initially up-regulates IL-12Rβ2 on naive $CD4^+$ T cells through enhancement of T-bet expression and then IL-12 strongly induces Th1 differentiation. However, the augmentation of Th17 differentiation by IL-6 and IL-23 was revealed to depend on TGF-β produced endogenously by IL-6 as well. Because IL-6 alone was not enough to induce Th17 differentiation, the level of TGF-β produced endogenously is considered to be less than that necessary for the induction of Th17 differentiation in synergy with IL-6. Even under these conditions, IL-6 and IL-23 efficiently augmented Th17 differentiation in TGF-β-dependent mechanism. Therefore, under certain circumstances where bioactive TGF-β production is limited, the combination of IL-6 and IL-23, both of which are largely produced by infection, may play an important role for the induction of Th17 differentiation in TGF-β-dependent mechanism.

Acknowledgments

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