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Human Th2 but Not Th9 Cells Release IL-31 in a STAT6/NF-κB–Dependent Way

Elisabeth Maier,* Dagmar Werner,*† Albert Duschl,* Barbara Bohle, † and Jutta Horejs-Hoeck*

IL-31, a member of the IL-6 protein family, is one of the latest additions to the list of T cell–derived cytokines. Th2 cells are regarded as a main source of IL-31, which is produced in response to stimulation by IL-4. Because the development of Th9 cells also requires IL-4 as a polarizing cytokine, the current study investigates IL-31 production in human Th9 cells compared with Th2 cells. We found that, although Th9 cells were able to release IL-31 during the first weeks of in vitro polarization, no IL-31 was detected in Th9 cultures after a final restimulation in the absence of polarizing cytokines. We further show that TGF-β, which is required to obtain Th9 cells in vitro, potently inhibits the release of IL-31 from Th2 cells, whereas IL-33, a cytokine associated with Th2-mediated inflammation, synergizes with IL-4 in inducing IL-31 secretion. To analyze the molecular mechanisms underlying the induction of IL-31, EMSAs, reporter gene assays, and small interfering RNA-based silencing experiments were carried out. We show that STAT6 and NF-κB are central players in mediating IL-31 expression induced by IL-4/IL-33. In addition, we identified a novel NF-κB–binding element within the IL31 promoter that mediates the enhancing effects of IL-33 on IL-4/STAT6–induced IL-31 expression in human Th2 cells. Taken together, this study shows that IL-4 is essential for the production of IL-31, whereas TGF-β significantly suppresses IL-31 expression at the mRNA and protein levels. As a consequence, in vitro polarized Th2 cells, but not Th9 cells, are able to release IL-31. The Journal of Immunology, 2014, 193: 645–654.

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D4+ Th cells are crucial players in orchestrating adaptive immune responses to various infectious agents. Depending on the type of presented Ag, naive CD4+ T cells develop into several distinct subsets that can be distinguished by their function and their unique cytokine profile (1, 2). More than 25 years ago, the first two subsets, termed Th1 and Th2, were described by Mosmann et al. (3). Th1 cells predominantly secrete IFN-γ and are important for protective immune responses to intracellular bacterial and viral infection. In contrast, Th2 cells contribute to the defense against helminthic parasites and are key players in allergic inflammation. Th2 cells are characterized by the production of IL-4, IL-5, and IL-13, which promote IgE/ eosinophil-mediated immune responses. More recently, two further subsets, known as Th17 cells and T regulatory (Treg) cells, were described. Through the production of IL-17 (IL-17A), IL-17F, IL-22, and IL-26, Th17 cells control immune responses to extracellular bacteria and fungi (4). In addition, naive CD4+ T cells can be induced to differentiate into inducible Treg (iTreg) cells, which are characterized by the production of IL-10 and TGF-β. Together with naturally occurring Treg cells, these cells are potent players in maintaining immune tolerance and the regulation of lymphocyte activation (1).

One of the latest additions to the list of CD4+ T cell subsets is the Th9 cell. This cell type develops in the presence of TGF-β and IL-4 and is characterized by the secretion of IL-9 (5, 6). Besides both being activated in the presence of IL-4, Th2 and Th9 cells also share functional features. Similar to Th2 cells, Th9 cells play an important role in promoting allergic responses and are involved in intestinal responses to helminths (7–9). Moreover, the expression of IL-9 was shown to be increased in asthmatic patients and atopic individuals compared with healthy subjects (10–12). These data indicate that IL-9 and Th2-derived cytokines may either cooperate or complement each other. Another cytokine that is tightly associated with allergic inflammation is IL-31. This type I cytokine was identified several years ago as a ligand for a heterodimeric receptor complex composed of the IL-31Rα (IL-31RA) chain and oncostatin M receptor β (13). IL-31 is predominantly secreted by activated CD4+ T cells (13). In particular, IL-31+ skin-homing, cutaneous lymphocyte Ag–positive T cells (14) are thought to be the main source of enhanced IL-31 expression, which has been observed in patients suffering from atopic dermatitis (AD) and acute allergic contact dermatitis (15). In line with these findings, it was reported that overexpression of IL-31 in a transgenic mouse model induced a severe skin phenotype resembling AD in humans (13). In addition, high IL-31 levels have been detected in sera and PBMCs from patients with allergic asthma, and IL-31 expression was positively correlated with severity of the disease (16). Together, these data indicate that IL-31 is associated with type 2 inflammation. A recent study clearly showed that IL-4 is the critical factor stimulating the release of IL-31. In the presence of IL-4, not only Th2 cells, but also Th1 cells, were able to release IL-31 (17). Because IL-4 is essential not only for the differentiation of Th2 cells, but also plays a critical role in Th9 development, the current study investigated IL-31 expression in different human in vitro polarized Th cell subsets, including Th9.
We show that, although Th9 cells secrete IL-31 during early T cell development, fully differentiated Th9 cells are not able to release IL-31. Downregulation of IL-31 was linked to the Th9-promoting cytokine TGF-β. Yet, the release of IL-31 by Th2 cells can be enhanced by addition of IL-33 and is mediated by the transcription factors STAT6 and NF-kB.

Materials and Methods

**Th cell polarization and ELISA**

All studies involving human cells were conducted in accordance with the guidelines of the World Medical Association’s Declaration of Helsinki. Human PBMCs were isolated from buffy coats of healthy donors by means of density gradient centrifugation using lymphocyte separation medium (PAA, Pasching, Austria). Naïve CD4+ T cells were isolated from PBMCs using the naïve CD4+ T cell isolation kit II (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in IMDM, containing 5% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all purchased from PAA) at a density of 2 x 10^6 cells/ml in 48-well plates precoated with anti-CD3 (clone OKT3; eBioscience, Vienna, Austria; coating concentration 1 μg/ml in 0.1 M Tris HCI [pH 9.5]) and in the presence of soluble anti-CD28 (2.5 μg/ml; BD Pharmingen, Heidelberg, Germany). For Th1 differentiation, 20 ng/ml IL-12 (Immunotools, Friesoythe, Germany) and 1 μg/ml anti-IL-4 (eBioscience, Vienna, Austria) were added to the cells. Th2 development was induced via the addition of 50 ng/ml IL-4 (gift of Novartis, Vienna, Austria) and 1 μg/ml anti-IL-12/anti-IL-23 (eBioscience). For Th9 cell generation, additionally 10 ng/ml TGF-β1 (PeproTech, London, U.K.) was used. Th17 cells were generated by adding 10 ng/ml IL-1β (Immunotools), 30 ng/ml IL-6 (Immunotools), 0.5 ng/ml TGF-β1 (PeproTech), 50 ng/ml IL-21 (Immunotools), and anti-IL-4. Treg cells were induced by 10 ng/ml TGF-β1 and 10 ng/ml IL-10 (Immunotools) with blocking Abs directed against IL-12/IL-23 and IL-4. Cells were cultured for 7 d, restimulated under the same conditions for an additional 7 d, and then reactivated by addition of anti-CD3/anti-CD28 for 48 h. Supernatants for cytokine secretion analyses by means of sandwich ELISA were taken after each stimulation period.

**Allergen-specific T cell clones**

Supernatants from T cell clones (TCC) specific for the major birch pollen allergen Bet v 1 were used for the assessment of IL-31. TCC were generated according to published protocols (18) and stimulated with autologous irradiated PBMCs and 5 μg/ml Bet v 1 (Biomay, Vienna, Austria) for 48 h. Cultures containing TCC and PBMCs in medium alone served as negative controls. Supernatants were kept at −20°C until cytokine measurements.

**Preparation of nuclear extracts and EMSAs**

Nuclear extracts from untreated and IL-4–induced CD4+ T cells or uninduced and IL-33–treated Th2 cells were prepared according to the method of Andrews and Faller (19). EMSAs were carried out as described previously (20, 21). Double-stranded oligonucleotides corresponding to the sequences −437 to −400, −282 to −241, and −160 to −118 relative to the start ATG from the human IL31 gene were generated by annealing synthetic sense and antisense oligonucleotides for 2 h, during which the temperature was gradually reduced from 95°C to 25°C, followed by radioactive 32P end labeling with [32P]dCTP (Hartmann Analytic, Braunschweig, Germany) using Klenow fragment (Fermentas, St. Leon-Roth, Germany). Labeled oligonucleotides were purified using the oligonucleotides are given as follows ( consensus nucleotides underlined; mutations in lower case): NF-κB −437 to −400 wild type (WT) sense, 5′-GGGCATTCTTCCTCCAGAAGATCCGTTGCG-3′ and antisense, 5′-GCCGCACAGGAAATTTGCGGGAGAAATGCAGAA-3′; NF-kB sense, 5′-GCGTTCGATTTCCCAGAAGATCCTGGTGCG-3′ and antisense, 5′-GGCCGACAGGAATTTGCGGGAGAAATGCAGAA-3′; STAT6 −282 to −241 WT sense, 5′-GGATGCATTGTCAGCCATGTTGCAATCG-3′ and antisense, 5′-GGGTGCAGACAACATCACTATCTCACAAATAGGCGAT-3′; STAT6 −160 to −118 WT sense, 5′-GAGGTGTGCAGACAACATCACTATCTCACAAATAGGCGAT-3′ and antisense, 5′-GGCCGATGCAAAGACATTTGCTTCTCCATGGAATAAGG-3′.

**Cloning of I31 promoter constructs**

A 474-bp fragment comprising the sequence −535 to −62 relative to the transcriptional start site of the human I31 promoter was amplified from human genomic DNA (Roche, Vienna, Austria) using Pfu polymerase with appropriate buffer (Fermentas) and the primers with attached restriction sites for MluI (forward primer) and XhoI (reverse primer) listed below. The PCR tube contained 36 μl H2O, 5 μl 10× Pfu buffer with MgSO4, 4 μl DMSO, 1 μl dNTPs (10 mM each), 2 μl genomic DNA, 1 μl forward and reverse primer (10 μM each), and 1 μl Pfu polymerase. PCR (5-min initial denaturation at 95°C, followed by 37 cycles of 15 s at 95°C, 30-s annealing at 60°C, and 5-min elongation at 72°C, and a final elongation step of 10 min at 72°C) was run on an Eppendorf Mastercycler (Eppendorf, Vienna, Austria). The PCR product was cloned into the pGL3 Basic luciferase reporter-gene vector (Promega, Mannheim, Germany). Site-directed mutagenesis of STAT6 sites and the NF-κB binding site was carried out by inverse PCR using the 5′-phosphorylated primers listed below. The sequences of all constructs were verified by sequencing at MWG (Ebersberg, Germany). The plasmids were used to transform chemocompetent Escherichia coli TG1 and purified using an EndoFree Plasmid Maxi Kit from Qiagen (Venlo, Netherlands). Sequence of the primers are as follows (restriction sites underlined; mutated nucleotides in lower case): IL31 474 bp MluI sense, 5′-ATGCGGTGAGCACCACCTCACACCAGTTA-3′; IL31 474 bp XhoI antisense, 5′-AGTCCTCGAGACGTCGATATATACAAAGGCGGC-3′; IL31 STAT6 −153 to −144 mut sense, 5′-GTTGAGAAAGATCGTATGAAATTGTTTCT-3′ and antisense, 5′-AAACACTTCAAATGTTCTGACCAAGCGGACG-3′; STAT6 −266 to −257 mut sense, 5′-GTTTGAAGATCTGTTGACCAAGCAG-3′ and antisense, 5′-AGGAGCATGAAATGCTTACGTCATGG-3′.

**Reporter gene assays**

The day before transfection, 1.25 x 10^5 HEK293 IL-4/L-13 cells (InvivoGen, Eubio, Vienna, Austria) were seeded into 24-well cell-culture plates in 1 ml DMEM supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids (all purchased from PAA), 100 μg/ml zeocin, and 10 μg/ml blasticidin, and incubated at 37°C in a humidified atmosphere containing 5% CO2. Cells were transfected with 1 μg luciferase reporter plasmid, 0.125 μg transmembrane form of ST2 (ST2L) expression construct (22) (provided by S. J. Martin, Dublin, Ireland), or empty pEF-Bos vector (23) (gift of S. Nagata, Kyoto, Japan) by means of calcium phosphate coprecipitation, as described previously (24). The day after the transfection, the medium was changed and cells were induced with 50 ng/ml IL-4 and/or 30 ng/ml IL-33 (PeproTech) or left unstimulated for 24 h, before luciferase activity was assessed.

**Small interfering RNA-based silencing**

Naïve CD4+ T cells were isolated and differentiated toward a Th2 phenotype, as described above. After 8 d of differentiation, cells were transfected with 100 pmol small interfering RNA (sRNA)-targeting STAT6 (Invitrogen stealth RNA, forward, 5′-CACAAGGACCAUUCCUGUGG- GACAA-3′ and reverse, 5′-UUUGUCACACGAGAUGUGCCUUUUGG-3′) to the natural promoter (n-type) or to the natural promoter (n-type) plus the control siRNA Control plasmid to the human cell T cell nuclease factor kit (Lonza, Szabo Scandic, Vienna, Austria), as described before (25), and then left incubating for 3 d in medium containing 100 U/ml IL-2
RNA isolation and quantitative real-time RT-PCR

Total RNA from cells was isolated using TRI Reagent (Sigma-Aldrich) and reverse transcribed with RevertAid H Minus M-MulV reverse transcriptase (Fermentas), according to the manufacturer’s instructions. Quantitative real-time PCR was carried out on a Rotorgene 3000 (Corbett Research, Mortlake, Australia) using 2 × q SYBR Green Supermix (Bio-Rad) and the primers listed below. The transcript for the large ribosomal protein P0 (RPL P0) was used as a reference. The specificity of the PCRs was checked by recording a melting curve for the PCR products. Relative mRNA expression levels were calculated using the formula:

\[ \frac{\text{Ct}_{	ext{target}} - \text{Ct}_{	ext{RPL P0}}}{\text{Ct}_{	ext{RPL P0}}} \]  

where \( \text{Ct} \) signifies the difference between the Ct values of the gene in question and the Ct value of the reference gene RPL P0. The primer sequences are as follows:

- **RPL P0 sense, 5’**-
  - GGTCGGTACCCCTCCAGGAAG-3’
- **RPL P0 antisense, 5’**-
  - GGATGGCGGGT-3’

- **GGACACGCAGTACAGCAAG-3’** and antisense
- **GGTTCCTCCTGTCTCAGG-3’** and antisense

**RPL P0** was used as a reference. The specificity of the PCRs was checked by recording a melting curve for the PCR products. Relative Ct values were calculated using the formula:

\[ D_{Ct} = D_{Ct,w h e n} - D_{Ct, w h e r e} \]

where \( D_{Ct} \) represents the threshold cycle of a given gene and \( D_{Ct,w h e n} \) and \( D_{Ct, w h e r e} \) represent the thresholds of the gene at two different conditions. The statistical significance of the differences was determined using ANOVA/Dunnett’s multiple comparisons test. 

SDS-PAGE and Western blotting

Cells were harvested by centrifugation, lysed in 2× Laemmli sample buffer (Bio-Rad), and frozen at \(-75^\circ C\). After thawing, lysates were denatured by 7-min incubation at 95°C and afterward centrifuged to remove the cell debris. Protein lysates were separated on precast NuPAGE 4–12% gradient gels (Invitrogen, Lofer, Austria) and blotted onto nitrocellulose membranes in a Transblot semidyblotting chamber (both Bio-Rad) using 2× transfer buffer (Invitrogen). The membrane was blocked by incubation in TBS containing 0.1% Tween 20 and 5% nonfat dry milk for 1 h. The primary Abs (anti-STAT6 [7Y], anti-STAT3, anti-STAT5, anti-GATA-3, anti-PSMAD2 [Ser465/467] and anti-PSMAD3 [Ser423/425], anti-SMAD2/SMAD3) and HRP-linked secondary Abs were purchased from Cell Signaling Technology (Danvers, MA) and used according to the manufacturer’s instructions. De-
tection was carried out using Supersignal ECL substrate (West Pico; Pierce, Rockford, IL) and Biomax x-ray films and exposure cassettes with intensifying screens (Kodak; Sigma-Aldrich) or the Immun-star WesternC chemiluminescence kit and the ChemiDoc MP Imaging system with Image Lab Software (all Bio-Rad). For stripping, the membrane was incubated in 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10 mM 2-ME for 20 min at 50°C.

**Results**

**IL-31 is secreted by developing but not by fully differentiated Th9 cells**

IL-31 was found to be associated with Th2-mediated diseases and hence is considered to be especially involved in Th2-driven immunity. In line with these observations, a recent study nicely showed that IL-4 is a key inducer of IL-31 expression in human CD4+ T cells (17). Because IL-4 is not only the main cytokine inducing the Th2 phenotype, but is also required for Th9 development (5, 6), we performed detailed analyses of IL-31 expression in different human T cell subsets, with a special focus on Th2 and Th9 cells. Naive CD4+ T cells were isolated from buffy coats and cultured under Th1-, Th2-, Th9-, Th17- and iTreg-polarizing conditions. After 1 wk of stimulation, cells were restimulated for another week under the same polarizing conditions as before. Thereafter, cells were activated for 2 d with Abs directed against CD3 and CD28. Supernatants were taken after each stimulation step and analyzed for IL-31 protein by ELISA. As shown in Fig. 1A, already after the first round of stimulation (day 7), IL-31 was predominantly secreted by Th2 cells, but developing Th9 cells secreted substantial amounts of IL-31 as well. Although secretion of IL-31 is generally enhanced after the second stimulation (day 13), the amount of IL-31 released by Th9 cells was clearly less than that secreted by Th2 cells. However, after the last restimulation (day 15), which was performed in the absence of the polarizing cytokines IL-4 and TGF-β, only Th2 cells, but not Th9 cells, released IL-31. To control for the quality of effector phenotype generation, signature cytokines of each subset were measured as well (Fig. 1B).

**FIGURE 2.** IL-4 and IL-33 promote the expression of IL-31 in human Th2 cells. (A) Naive CD4+ T cells were polyclonally activated by anti-CD3/anti-CD28 and treated with IL-4 in rising concentrations, as indicated. After 1 wk of stimulation, cells were restimulated for 7 d. Secretion of the cytokines IL-31, IL-5, and IL-13 was measured by ELISA. Data represent mean values of seven independent experiments carried out in duplicates using cells from seven individual donors. The black horizontal bars symbolize the overall mean for each experimental group. (B) Naive CD4+ T cells were differentiated toward Th2 cells in the presence of rising concentrations of IL-33. Cytokine secretion was analyzed by ELISA. Mean values of duplicates from seven independent experiments are shown. The black horizontal bars signify the overall means. Statistical analyses: one-way ANOVA/Dunnett’s multiple comparisons test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**FIGURE 3.** TGF-β blocks the release of IL-31 by Th2 cells. (A) Naive CD4+ T cells were cultured with anti-CD3/anti-CD28 + IL-4 and treated with TGF-β1 in concentrations as indicated to induce a gradual switchover from the Th2 phenotype toward the Th9 phenotype. After 1 wk in culture, cells were restimulated under the same conditions. Protein expression of IL-31 and IL-9 after 1 wk of restimulation was assessed by ELISA. Mean values of duplicates from seven experiments using cells from individual donors are shown (black horizontal bars signify the overall means; statistical analyses: one-way ANOVA/Dunnett’s multiple comparisons test, *p ≤ 0.05). (B) Naive CD4+ T cells were cultured under Th2-polarizing conditions and restimulated under Th2- or Th9-promoting conditions for 6 d. Expression of Il31, Il9, Stat6, and Spi1 was analyzed by quantitative RT-PCR. Data show mean values of at least six experiments; error bars indicate SDs. Statistical analyses: t test. *p ≤ 0.05.
To test whether our observation on IL-31 release by in vitro primed Th cell subsets holds true for in vivo primed and differentiated T cells, we analyzed the production of IL-31 by 13 allergen-specific TCC expanded from the peripheral blood of allergic patients. TCC were classified into Th2- and Th0-like cells according to their allergen-induced production of IL-4 and IFN-γ (18). Th2 clones were further distinguished by their IL-4 expression (IL-4high Th2, IL-4low Th2). We observed IL-9 secretion by four Th2-like clones (ratio IL-4/IFN-γ > 5) and five Th0-like clones (ratio IL-4/IFN-γ = 0.2–5). However, neither of the allergen-specific TCC showed a clear Th9-like phenotype. Supernatants from these nine TCC and the four IL-9–negative Th2 clones were subjected to IL-31 ELISA. The production of IL-31 correlated with the release of IL-4, but not with IL-13 or IL-9 (Fig. 1C). Hence, among the Th2 clones, only those cultures that secreted high levels of IL-4 produced substantial amounts of IL-31. This clearly substantiates the observation that IL-4 is a key factor in promoting IL-31 expression.

IL-4 and IL-33 drive the secretion of IL-31 in (developing) Th2 cells in a dose-dependent fashion

Because IL-4–dependent Th cell subsets were the only sources of IL-31 in our experiments, we sought to analyze the effect of IL-4 on IL-31 production in more detail. Thus, we isolated naive CD4+ T cells from human buffy coats and induced differentiation of Th2 cells by exposing the CD4+ T cells to rising concentrations of IL-4. After 1 wk, cells were restimulated and IL-31 released into the supernatant was measured after 7 d of restimulation. As controls, the levels of the Th2 cytokines IL-5 and IL-13 were also determined. ELISA analyses revealed that IL-4 increases the expression of IL-31 along with IL-5 and IL-13 in a concentration-dependent manner (Fig. 2A).

The IL-1 family member IL-33 is associated with Th2-type immune reactions and has been shown to augment the expression of the Th2-secreted cytokines IL-13 and IL-5 in murine Th2 cells (26, 27). The heterodimeric receptor for IL-33 consists of ST2L, also known as IL-1R–like 1, and IL-1R accessory protein (26, 28). Whereas IL-1R accessory protein is ubiquitously expressed, ST2L is predominantly found on the surface of mast cells and Th2 cells (29). Th2 cells acquire ST2L expression in the course of differentiation, and IL-33 itself was shown to promote the expression of ST2L by Th2 cells (27). To analyze whether IL-33 impacts on IL-31 expression by human in vitro generated Th2 cells, naive CD4+ T cells were skewed toward a Th2 phenotype in the presence of IL-33 in rising concentrations, ranging from 3 to 100 ng/ml. Analyses of IL-31, IL-5, and IL-13 expression by ELISA revealed similar data to that observed in the murine system (26, 27): production of IL-5 and IL-13 increased with rising concentrations of IL-33. Although the effect was less pronounced, the same tendency was observed for IL-31 (Fig. 2B).

TGF-β attenuates IL-31 secretion by Th2-polarized cells

Although IL-4 and IL-33 are known to promote the Th2 phenotype, CD4+ T cells cultured in the presence of IL-4 and TGF-β clearly develop toward Th9. Because we observed lower IL-31 levels in Th9– compared with Th2-skewed cells, we speculated that this is due to the addition of TGF-β. To prove this hypothesis, we stimulated naive CD4+ T cells under Th2-polarizing conditions

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**FIGURE 4.** Phosphorylation of SMAD2 and SMAD3 correlates with limited IL-31 production. (A) Naive human CD4+ T cells derived from five individual donors were analyzed for the expression of TGFβ, TGFβRI, and TGFβRII mRNA by real-time quantitative RT-PCR. (B) A fraction of the cells was in vitro differentiated into Th2 and Th9 cells. IL-31 secretion was analyzed by ELISA on day 13. Data represent mean values of duplicates, and error bars indicate SDs. (C) Naive CD4+ T cells were stimulated for 15 min with TGF-β1 at the concentrations indicated. Phosphorylation of SMAD2 and SMAD3 (p-SMAD2/3) and total SMAD2/3 was monitored by Western blotting. Phosphorylation levels relative to total SMAD2/3 and normalized to the signal maximum are shown.
and treated them with TGF-β in rising concentrations to achieve a gradual switchover toward Th9. ELISA analyses showed that TGF-β increases the secretion of IL-9 while simultaneously decreasing the release of IL-31. Additionally, the release of IL-5 and IL-13 was inhibited by TGF-β, indicating that there occurred a transition from the Th2 to the Th9 phenotype (Fig. 3A). One of the key transcription factors in Th9 development is PU.1, the gene product of Spi1 (30). Because expression of PU.1 is induced by TGF-β (31), we investigated whether the TGF-β–mediated suppression of IL-31 is directly associated with the induction of Spi1/PU.1. Therefore, we stimulated naive CD4+ T cells under Th2-polarizing conditions for 1 wk and restimulated them under either Th2-polarizing or Th9-inducing conditions for 6 d. In accordance with the protein data, we observed a clear downregulation of IL-31 mRNA expression in cells cultured in the presence of TGF-β, whereas IL-9 and Spi/PU.1 mRNA expression was strongly up-regulated in Th9 cells. As expected, STAT6 expression was only moderately affected by TGF-β treatment (Fig. 3B).

**SMAD2/3 phosphorylation in naive CD4+ T cells results in diminished IL-31 production**

Because IL-31 production by Th2 and developing Th9 cells varied greatly from donor to donor, we analyzed mRNA expression of TGF-β and its receptor complex composed of TGFBR I and II in naive human T cells (n = 5) before they were subjected to Th2 and Th9 differentiation. Although the differences in mRNA expression of TGF-β and its receptor between individual donors were rather low (Fig. 4A), TGFBRI and TGFBRII seemed to be negatively correlated with IL-31 production in Th2 cells (Fig. 4B). Additionally, a fraction of the naive cells was treated for 15 min with TGF-β1 in rising concentrations to monitor potential differences in TGF-β downstream signaling. TGF-β1 signaling is central to T cell homeostasis and maintenance of the naive T cell compartment. Hence, freshly isolated human T cells exhibit active TGF-β downstream signaling, which results in the phosphorylation of the signaling molecules SMAD2 and SMAD3 (32). In agreement with this, phospho-SMAD2/3 was detected in samples from untreated cells. However, the SMAD phosphorylation level in untreated cells did vary between the donors and seemed to determine the ability of the cells to produce IL-31. We found that cells that had shown moderate SMAD phosphorylation right after isolation secreted more IL-31 than cells with strong SMAD phosphorylation (Fig. 4C).

**STAT6 and NF-kB bind to the human Il31 genomic locus**

IL-4–induced gene expression is mainly mediated by the transcription factor STAT6, whereas IL-33 activates NF-κB (26). Analysis of the human Il31 promoter sequence revealed the presence of two STAT6 consensus sequences, located 257 and 144 bp upstream of the translation start site and one NF-κB consensus motif at position −409 relative to the translation start site (Fig. 5A). These motifs were tested in gel-shift assays for the ability to bind STAT6 or NF-κB.

![Figure 5](http://www.jimmunol.org/)  
**Figure 5.** STAT6 and NF-κB bind to response elements within the human Il31 promoter. (A) Schematic representation of the human Il31 genomic locus. Positions of the two STAT6 binding sites and the NF-κB response element are indicated relative to the translational start site (ATG). (B) Gel-shift assays using radiolabeled probes comprising the NF-κB response element (left panel) and the two STAT6 binding sites (right panel) were carried out. Nuclear extracts from untreated or IL-33–induced in vitro generated Th2 cells, or from uninduced or IL-4–stimulated naive human T cells, were incubated with double-stranded oligonucleotide harboring the WT transcription factor–binding motifs or mutated versions thereof (mut), and the formed nucleoprotein–DNA complexes were resolved by native PAGE. For competition assays, 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. In supershift experiments, nuclear extracts were preincubated with Abs directed against the NF-κB subunits p50, p52, and p65 or Abs specific for STAT6 or STAT5. Black arrows indicate the positions of the supershifted bands.
The human IL31 promoter is cooperatively activated via the proximal STAT6 binding site and a NF-κB motif

To determine the contributions of the three transcription factor binding sites identified by EMSA to regulating the human IL31 promoter, we amplified a 474-bp fragment comprising the IL31 promoter sequence –535 to –62 relative to the start ATG from human genomic DNA and cloned it into the pGL3 Basic luciferase reporter-vector. By site-directed mutagenesis, the same mutations that abrogated complex formation in the EMSA were introduced into one or both STAT6 binding sites. Additionally, the NF-κB binding site was mutated (Fig. 6A). The resulting constructs were transfected into the HEKblue IL-4/IL-13 cell line. These cells stably express STAT6 and hence are IL-4 responsive. To be able to study the effects of IL-33 on the IL31 promoter, cells also have to be transfected with the IL-33R subunit ST2L. We therefore cotransfected the reporter plasmid containing the WT IL31 promoter with an expression plasmid encoding human ST2L (22) or an empty pEF-Bos vector (23) and induced the cells with 50 ng/ml IL-4 and/or 30 ng/ml IL-33. After 24 h of stimulation, luciferase activity was assessed. Data represent mean values of three independent experiments carried out in duplicates; error bars indicate SDs. ALU, arbitrary light units; ui, uninduced. (C) Reporter gene assay of HEKblue IL-4/IL-13 cells transfected with the WT IL31 promoter construct and either an expression vector encoding the IL-33R subunit ST2L or empty pEF-Bos vector. The day after the transfection, cells were induced with 50 ng/ml IL-4 and/or 30 ng/ml IL-33. After 24 h of stimulation, luciferase activity was assessed. Data represent mean values of two independent experiments carried out in duplicates are shown. Error bars indicate SDs. Statistical analyses: one-way ANOVA/Tukey’s multiple comparisons test. ****p ≤ 0.0001.

FIGURE 6. Mutation of STAT6-binding elements within the IL31 promoter impairs promoter activity. (A) Schematic representation of reporter constructs from the human IL31 promoter. A 474-bp fragment of the human IL31 promoter was cloned into the pGL3 Basic luciferase reporter plasmid. Mutations were introduced by site-directed mutagenesis into the NF-κB binding site or into one or both STAT6-binding elements. (B) Reporter gene assay of HEKblue IL-4/IL-13 cells transfected with the WT IL31 promoter construct and either an expression vector encoding the IL-33R subunit ST2L or empty pEF-Bos vector. The day after the transfection, cells were induced with 50 ng/ml IL-4 and/or 30 ng/ml IL-33. After 24 h of stimulation, luciferase activity was assessed. Data represent mean values of three independent experiments carried out in duplicates; error bars indicate SDs. ALU, arbitrary light units; ui, uninduced. (C) Reporter gene assay of HEKblue IL-4/IL-13 cells transfected with the WT IL31 promoter construct and either an expression vector encoding the IL-33R subunit ST2L or empty pEF-Bos vector. The day after the transfection, cells were induced with 50 ng/ml IL-4 and/or 30 ng/ml IL-33. After 24 h of stimulation, luciferase activity was assessed. Data represent mean values of two independent experiments carried out in duplicates are shown. Error bars indicate SDs. Statistical analyses: one-way ANOVA/Tukey’s multiple comparisons test. ****p ≤ 0.0001.

IL-4-mediated IL-31 expression, whereas the distal STAT6 motif and the NF-κB site further enhance IL-31 expression.

STAT6 is required for IL-31 expression by human Th2 cells

To confirm the crucial role of STAT6 for IL-31 expression in human Th2 cells, mRNA expression in STAT6-silenced, in vitro generated Th2 cells was analyzed. Silencing of STAT6 was accomplished by transfecting the cells with siRNAs that target STAT6 mRNA for degradation. Three days after transfection, STAT6-silenced cells were restimulated for 24 h under Th2-polarizing conditions. The efficacy of STAT6 silencing was monitored by immunoblotting. Transfection of the cells with siRNA specific for STAT6 resulted in a radical reduction of STAT6 protein, observed by Western blot detection of phospho-STAT6 and STAT6 (Fig. 7A). To test the specificity of the silencing RNA, we also measured STAT3. STAT3, like STAT6, has been shown to contribute to the Th2 phenotype (34), but STAT3 levels were not affected by the STAT6-interfering oligonucleotide (Fig. 7A). When we detected GATA-3, the master regulator of Th2 cells, we found it to be present not only in control cells, but also in STAT6-silenced cells (Fig. 7A). Real-time quantitative RT-PCR analyses revealed that, after transfection, both samples showed similar expression of GATA-3.
but upon restimulation, only the control-transfected cells were able to upregulate GATA-3 mRNA (Fig. 7B). Analyses of mRNA expression of the Th2 cytokine genes Il4, Il5, and Il13 showed that silencing of STAT6 reduced the levels of Il5 and Il13 transcripts in restimulated cells, whereas Il4 expression was not significantly impaired (Fig. 6B). Restimulation under Th2 conditions clearly induced the expression of Il31 mRNA in control cells, but not in STAT6-silenced cells (Fig. 7B), which demonstrates that STAT6 is absolutely essential for IL-31 expression.

Discussion

Although numerous in vitro and in vivo studies have aimed at investigating the role of IL-31, its biological functions are still not fully understood. Human studies and overexpression studies in IL-31 transgenic mice clearly indicate that high IL-31 concentrations are tightly associated with allergic asthma and inflammatory skin diseases, including AD. Thus, IL-31 is regarded as a novel player associated with the activation of numerous diseases, including asthma and AD (40), was shown to promote IL-4–driven IL-31 secretion in a concentration-dependent fashion. This observation substantiates a recent study that reports enhanced Il31 mRNA expression in IL-33–treated memory T cells from grass pollen–secreting cells, while freshly isolated human T cells (32). We found SMAD activation to be negatively correlated with the potential of the cells to produce IL-31, which indicates that SMAD2 and SMAD3 might play a role in IL-31 suppression. It is well known that, in Th2 effector cells, TGF-β induces the expression of PU.1, which interferes with GATA-3 DNA binding (36). Consequently, PU.1 negatively regulates the Th2 phenotype by inhibiting the expression of IL-5 and IL-13, both of which depend on GATA-3 (37, 38). Because IL-31 expression is mainly mediated by STAT6, it is unlikely that TGF-β dependence on IL-31 synthesis was consistently observed, but with high interindividual variability. Analysis of TGF-β signaling revealed that freshly isolated naive CD4+ T cells show moderate to strong phosphorylation of SMAD2 and SMAD3, which is in line with an earlier study showing SMAD2 and SMAD3 phosphorylation in freshly isolated human T cells (32). We found SMAD activation to be negatively correlated with the potential of the cells to produce IL-31, which indicates that SMAD2 and SMAD3 might play a role in IL-31 suppression. It is well known that, in Th2 effector cells, TGF-β induces the expression of PU.1, which interferes with GATA-3 DNA binding (36). Consequently, PU.1 negatively regulates the Th2 phenotype by inhibiting the expression of IL-5 and IL-13, both of which depend on GATA-3 (37, 38). Because IL-31 expression is mainly mediated by STAT6, it is unlikely that TGF-β dependence on IL-31 synthesis was consistently observed, but with high interindividual variability. Analysis of TGF-β signaling revealed that freshly isolated naive CD4+ T cells show moderate to strong phosphorylation of SMAD2 and SMAD3, which is in line with an earlier study showing SMAD2 and SMAD3 phosphorylation in freshly isolated human T cells (32). We found SMAD activation to be negatively correlated with the potential of the cells to produce IL-31, which indicates that SMAD2 and SMAD3 might play a role in IL-31 suppression. It is well known that, in Th2 effector cells, TGF-β induces the expression of PU.1, which interferes with GATA-3 DNA binding (36). Consequently, PU.1 negatively regulates the Th2 phenotype by inhibiting the expression of IL-5 and IL-13, both of which depend on GATA-3 (37, 38). Because IL-31 expression is mainly mediated by STAT6, it is unlikely that TGF-β dependence on IL-31 synthesis was consistently observed, but with high interindividual variability. Analysis of TGF-β signaling revealed that freshly isolated naive CD4+ T cells show moderate to strong phosphorylation of SMAD2 and SMAD3, which is in line with an earlier study showing SMAD2 and SMAD3 phosphorylation in freshly isolated human T cells (32). We found SMAD activation to be negatively correlated with the potential of the cells to produce IL-31, which indicates that SMAD2 and SMAD3 might play a role in IL-31 suppression. It is well known that, in Th2 effector cells, TGF-β induces the expression of PU.1, which interferes with GATA-3 DNA binding (36). Consequently, PU.1 negatively regulates the Th2 phenotype by inhibiting the expression of IL-5 and IL-13, both of which depend on GATA-3 (37, 38). Because IL-31 expression is mainly mediated by STAT6, it is unlikely that TGF-β dependence on IL-31 synthesis was consistently observed, but with high interindividual variability. Analysis of TGF-β signaling revealed that freshly isolated naive CD4+ T cells show moderate to strong phosphorylation of SMAD2 and SMAD3, which is in line with an earlier study showing SMAD2 and SMAD3 phosphorylation in freshly isolated human T cells (32). We found SMAD activation to be negatively correlated with the potential of the cells to produce IL-31, which indicates that SMAD2 and SMAD3 might play a role in IL-31 suppression. It is well known that, in Th2 effector cells, TGF-β induces the expression of PU.1, which interferes with GATA-3 DNA binding (36). Consequently, PU.1 negatively regulates the Th2 phenotype by inhibiting the expression of IL-5 and IL-13, both of which depend on GATA-3 (37, 38).
vation by IL-4 and IL-33 and its suppression by TGF-β. IL-31 seems to behave like a classical Th2 cytokine. However, at the transcriptional level, IL-5 and IL-13 are regulated by GATA-3 (37, 38, 41), whereas IL-31 seems to fully depend on STAT6. Because within CD4+ T cell subsets GATA-3 expression is limited to Th2 cells, the expression of GATA-3–dependent genes is also restricted to Th2 cells. By contrast, STAT6 is widely expressed. Hence, cells other than Th2 can be stimulated to produce IL-31, as was shown for Th1 cells. Th1 clones produce IL-31, but not IL-13, in response to IL-4. Moreover, despite being exposed to IL-4, the amount of secreted IFN-γ did not alter, which indicates that the cells retain their Th1 character (35). One might assume that IL-31, due to its direct dependence on IL-4/STAT6 signaling, differs from the established Th2 cytokines IL-4, IL-5, and IL-13 also in its expression kinetics and function during allergic inflammation. Although there is convincing evidence that IL-31 induces proinflammatory cytokines, and there is a clear correlation between IL-31 level and disease severity in atopic skin disorders (13, 15, 42, 43), further observations are needed to elucidate the definite role of IL-31 in allergic inflammation.

It is well established that IL-4–induced gene regulation is mediated by the transcription factor STAT6, whereas IL-33 activates NF-kB. To identify the molecular mechanisms underlying IL-4/IL-33–induced IL-31 production, we analyzed the human Il31 locus for the presence of candidate transcription factor–binding motifs and found two STAT6 consensus sites and one putative NF-κB–binding motif. EMSAs showed that STAT6 present in nuclear extracts from IL-4–treated primary human T cells can associate with both STAT6 response elements, but especially the proximal STAT6 response element binds STAT6 with high affinity. Moreover, we showed for the first time, to our knowledge, binding of IL-33–activated NF-κB to the NF-κB response element within the human Il31 promoter. To evaluate the role of the transcription factor binding sites for Il31 promoter activity, reporter gene assays were carried out. HEKblue IL-4/IL-13 cells cotransfected with a luciferase reporter under the control of the Il31 promoter and an expression plasmid encoding the IL-33-33 subunit ST2L showed IL-4–dependent activation of the reporter, which was augmented in the presence of IL-33 and thus mirrored our results from human Th2 cells. Introduction of specific mutations into the NF-κB binding site and the distal STAT6–responsive motif of the Il31 promoter lowered, but did not completely abolish, its responsiveness. Of note, mutation of the NF-κB site and the STAT consensus sequence both seemed to impair enhancement of luciferase activity following IL-33 treatment. In contrast, mutation of the proximal STAT6 motif resulted in complete loss of inducibility. This demonstrates that the proximal STAT6 site is indispensable for IL-4–mediated Il31 transcription, whereas the distal STAT6 motif and the NF-κB site instead enhance Il31 gene expression. To define the role of STAT6 in IL-31 expression by human Th2 cells in more detail, we performed siRNA–mediated silencing of STAT6 in in vitro generated human Th2 cells. Restimulation of control-transfected cells under Th2 conditions resulted in an upregulation of Il31 mRNA, but failed to induce Il31 expression in STAT6-deficient cells. STAT6 knockdown also resulted in reduced mRNA levels of the Th2 cytokines Il5 and Il13 as well, but the effect was less pronounced. This may be explained by the fact that expression of IL-5 and IL-13 in Th2 cells mainly depends on the transcription factor GATA-3 throughout all developmental stages (41). Compared with control cells, GATA-3 mRNA was not affected by silencing of STAT6. However, STAT6–silenced cells failed to upregulate STAT6–3 expression upon restimulation with IL-4, because GATA-3 itself is regulated by STAT6 (44, 45). Hence, STAT6 silencing might indirectly impact on the expression of Il5 and Il13 by reducing the levels of GATA-3. In contrast to IL-5 and IL-13, IL-4 production by fully differentiated Th2 cells is independent of GATA-3 (41). We found no difference in Il4 expression levels between STAT6 knockdown and control cells.

Taken together, our study gives new insights into the regulation of IL-31 expression in human Th2 cell subsets, with a special focus on Th2 and developing Th9 cells.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


IL-31 EXPRESSION BY HUMAN IN VITRO POLARIZED Th CELLS


