Human IL-31 is induced by IL-4 and promotes T_H2-driven inflammation

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Background: The pruritic cytokine IL-31 has been shown to be expressed by murine activated effector T Lymphocytes of a T_H2 phenotype. Like IL-17 and IL-22, IL-31 is a tissue-signaling cytokine the receptor of which is mainly found on nonimmune cells. An overabundance of IL-31 has been shown in patients with atopic disorders, including dermatitis, as well as asthma, and therefore represents a promising drug target, although its regulation in the context of the human T_H2 clusters is not yet known.

Objective: We sought to address the gene regulation of human IL-31 and to test whether IL-31 possesses a similar proallergic function as members of the human T_H2 cytokine family, such as IL-4, IL-5, and IL-13.

Methods: Polyclonal and purified protein derivative of tuburculin–specific T-cell clones were generated. T_H phenotype was determined, and IL-31 was measured by means of ELISA. Gene expression of primary bronchial epithelial cells treated with IL-31 was also measured.

Results: IL-31 was expressed by all of the T_H2 clones and not by T_H1, T_H17, or T_H22. This expression was dependent on autocrine IL-4 expression from these clones because it could be reduced if blocking antibodies to IL-4 were present. Interestingly, T_H1 clones were able to express IL-31 if IL-4 was added to culture. This IL-31 expression was transient and did not affect the phenotype of the T_H1 clones. IL-31 was able to induce proinflammatory genes, such as CCL2 and granulocyte colony-stimulating factor.

Conclusion: IL-31 is not a T_H2 cytokine in the classical sense but is likely to be expressed by a number of cells in an allergic situation in which IL-4 is present and possibly contribute to the allergic reaction. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: *Allergic airway inflammation, T_H2 cells, IL-31, IL-4, normal human bronchial epithelial cells, IL-33, chromatin immunoprecipitation*

IL-31 is a recently described cytokine structurally related to IL-6 and oncostatin M.[1,2] Although its function is not fully understood, there is evidence that an overabundance of IL-31 is linked to allergic inflammation in the lung, gut, and, especially, skin. Transgenic mice engineered to overexpress IL-31 have atopic dermatitis–like symptoms, such as alopecia, pruritis, and skin inflammation.[1] Increased IL-31 levels have been detected in a number of inflamed tissues, including those from patients with atopic dermatitis,[3] atopic eczema,[4] and Crohn disease,[5] and in sera from patients with allergic asthma and rhinitis[6] and is therefore a promising drug target.

The IL-31 receptor consists of 2 subunits, the ubiquitously expressed oncostatin M receptor and the IL-31 receptor α (IL-31RA), which has more restricted expression. Similar to IL-13,[7] IL-17,[8] and IL-22,[9] the IL-31 receptor complexes are primarily expressed on nonhematopoietic tissue,[1] such as the skin, trachea, and endothelium, suggesting IL-31 has a number of functions regulating tissue responses. Treatment of lung epithelial cell lines with recombinant IL-31 induces inflammation-associated chemokines, such as CCL2 and CCL5, and wound repair factors, such as epidermal growth factor and vascular endothelial growth factor.[10] Recent studies demonstrate that IL-31 also regulates the differentiation of keratinocytes and the expression of filaggrin, which is known to play a critical role in the barrier function of the skin of patients with atopic dermatitis.[11] Interestingly, dorsal root ganglion cells express IL-31 receptor complex,[1,12] which might explain the high level of pruritus observed in mice when IL-31 is overexpressed in the skin and potentially could link IL-31 with airway hyperresponsiveness in asthmatic patients.

Initial studies have identified IL-31 as being predominantly expressed by T_H2 cells.[1] T_H2 cells are key players in the allergic response, migrating to the site of challenge to recruit and activate eosinophils, B cells, mast cells, and epithelial cells and switching antibody production to IgE through the actions of the cytokines IL-4, IL-5, IL-13, and IL-10. T_H2 polarization from naive T cells is initially induced by activated dendritic cells[13] or exogenous IL-4 from basophils.[14] T_H2 differentiation can also be induced by IL-25[15] or IL-33,[16] but once established, IL-4 is then produced autologously to maintain growth of the T_H2 culture.

This study aimed to investigate the expression of IL-31 from T cells to examine the potential role IL-31 might play in an allergic

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Abbreviations used

ChIP:	Chromatin immunoprecipitation
EF1a:	Elongation factor 1a
FACS:	Fluorescence-activated cell sorting
G-CSF:	Granulocyte colony-stimulating factor
IL-31RA:	IL-31 receptor α
NHBE:	Normal human bronchial epithelial cell
PPD:	Purified protein derivative of tuburculin
RT-PCR:	Real-time PCR

reaction. A preliminary investigation of IL-31 function was performed by using real-time PCR (RT-PCR) analysis of genes induced by IL-31 in bronchial epithelial cells.

METHODS

Methods for PBMC isolation, T-cell subset purification, and ELISA are described in the Methods section in this article's Online Repository at www.jacionline.org.

Subjects

All donors provided blood with informed consent to a study approved by the ethics committee of Royal Brompton & Harefield NHS Trust and the National Heart and Lung Institute. Atopic donors were all sensitized to timothy grass, as determined by positive skin prick test responses (wheal >3 mm and negative control) with standard reagents (ALK-Abelló, Hörsholm, Denmark) and allergen-specific IgE measurement. Healthy nonatopic donors were not allergic to any common allergen, as defined by negative skin prick test responses and negative IgE levels.

T-cell cloning

$CD4^+$ T cells were isolated as described in the Methods section in this article's Online Repository. The $CD4^-$ fraction was used as the source of feeder cells (irradiated to 4000 rads). T cells (0.6 per well) in a 96-well, U-bottom microtiter plate were cultured with 250,000 irradiated feeder cells per well plus 1 μg/mL PHA (Sigma-Aldrich, St Louis, Mo) and 20 ng/mL IL-2 (Novartis, Basel, Switzerland). After 10 days, clones were picked and fed 3 times a week with fresh media and IL-2. Clones were restimulated every 14 days with plate-bound anti-CD3 and anti-CD28. Cloning media comprised RPMI 1640 plus 10% FCS, 5% Human AB serum, nonessential amino acid solution, glutamine solution, 1 mmol/L sodium pyruvate, penicillin/streptomycin solution, 50 μmol/L β-mercaptoethanol (sera from Sigma-Aldrich and all other components from Invitrogen [Carlsbad, Calif], as before). Clones were characterized by means of ELISA: T_H1 clones were defined as IFN-γ high and IL-4, IL-17, and IL-22 low, and T_H2 clones were defined as IL-4 high and IFN-γ, IL-17, and IL-22 low. T_H17 and T_H22 cells were defined as expressing IL-17 only or IL-22 only, respectively. No T_H22 or T_H17 clones were generated from the first donor. See Fig E1 in this article's Online Repository at www.jacionline.org for validation of characterization.

Real-time PCR

Total RNA was isolated with RNeasy kits (Qiagen, Hamburg, Germany), RNA was reverse transcribed with Revertaid reverse transcription kits (Thermo Fisher, Waltham, Mass), and RT-PCR was carried out with SYBR-green PCR mix (Invitrogen) using a standard 40-cycle protocol. The housekeeping gene for T-cell PCR was elongation factor 1a, and for normal human bronchial epithelial cell (NHBE) PCR, 18s was used. Exon spanning primers were designed in house (Table I). The reaction was monitored and quantified in a 384-well 7900 HT FAST Realtime PCR system (Applied Biosystems, Foster City, Calif).

NHBE culture

NHBEs were obtained from Lonza (Basel, Switzerland) and maintained in the proprietary growth medium BEGM (Lonza). Cells were used while still proliferating at 70% confluence and never at a passage number of greater than 7. Before each assay, the media in each well were replaced with fresh media (500 μL per well of a 24-well plate).

IL-31RA staining

NHBEs were seeded onto round glass cover slips in 24-well plates and cultured in 500 μL of media. When cells reached 70% confluence, cover slips were removed, washed in PBS, and fixed for 20 minutes in 4% paraformaldehyde PBS (Sigma-Aldrich). Primary goat anti-human polyclonal IL-31RA (clone AF2769; R&D Systems, Minneapolis, Minn) was used at 1:100 dilution for 1 hour's incubation at room temperature. Staining was visualized with biotinylated horse anti-goat (1:170 dilution, Vector Laboratories, Burlingame, Calif) and streptavidin-conjugated Alexa Fluor 488 (1:400 dilution, Invitrogen). A control of secondary antibody only was used to demonstrate antibody specificity, and nuclei were counterstained during cover slipping with Vecta shield plus 4′-6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories).

Chromatin immunoprecipitation-sequencing

Chromatin immunoprecipitation (ChIP) was performed on native chromatin derived from *ex vivo* differentiated human healthy nonatopic T_H2 cells cultured and polarized for 4 weeks.[17] Chromatin was prepared, and ChIP was undertaken, as described by Rani et al.[18] The IL-31 gene within a 60-kb section of chromosome 12 was analyzed for monomethylation and trimethylation of lysines 4 and 27 of histone 3.

RESULTS

Allergen stimulation induces IL-31 mRNA expression

IL-31 has been detected in patients with atopic skin diseases,[3] such as dermatitis and eczema. Therefore we sought to determine whether IL-31 is produced during an *in vitro* stimulation of PBMC cultures with a common aeroallergen. PBMCs from nonallergic donors or donors with grass pollen allergy were incubated with recombinant Phl p 5 protein out of the dominant timothy grass pollen, a very common allergen for patients with allergic rhinitis in the United Kingdom.[19] After 7 days' culture with *Phleum pratense* protein, IL-31 expression was measured by using RT-PCR and found to be significantly ($P \leq .05$) higher (20.6 ± 11.89 atopic mean fold change [± SEM] vs 1.8 ± 1.06 nonatopic mean fold change) in the cultures from atopic donors compared with those from nonatopic donors (Fig 1, *A*). *P pratense* protein induced expression of IL-4 (Fig 1, *B*), with a trend toward increased expression by atopic donors (9.23 ± 2.32 atopic mean fold change increase [± SEM] vs 4.01 ± 2.22 nonatopic mean fold change increase). Purified protein derivative of tuberculin (PPD) was used as a control stimulation because PPD-specific T cells show a bias toward a T_H1 phenotype[20] in contrast to *P pratense*–specific T cells, which exhibit a T_H2 phenotype.[1] In addition, there should be no difference in PPD responses between atopic and nonatopic donors so long as the donor has been immunized against tuberculosis. PPD stimulation did not induce appreciable amounts of IL-31 or IL-4 from PBMCs from either atopic or nonatopic donors but did induce IFN-γ (3.35 ± 0.58 nonatopic mean fold change increase [± SEM] vs 5.77 ± 1.33 atopic mean fold change increase; Fig 1, *C*) from both types of donors, suggesting a T_H1 response, which is in agreement with the literature. Polyclonal T-cell receptor stimulation was used to provide a baseline T-cell

TABLE I. List of primer sequences

Gene	Forward	Reverse
IL-31	5′-GATGATGTACAGAAAATAGTCGAGGAATT-3′	5′-CTTCTCTTCCTCCACATCTTTCAAA-3′
IL-4	5′-AACGGCTCGACAGGAACCT-3′	5′-ACTCTGGTTGGCTTCCTTCCA-3′
IFN-γ	5′-TCTCGAAACGATGAAATATACAAGTT-3′	5′-GTAACAGCCAAGAGAACCCAAAA-3′
EF1a	5′-CTGAACCATCCAGGCCAAAT-3′	5′-GCCGTGTGGCAATCCAAT-3′
18s	5′- CAGCCACCCGAGATTGAGCA-3′	5′-TAGTAGCGACGGGGGGTGTG-3′
IL-8	5′-GAGTGATTGAGAGTGGACCACACT-3′	5′-GCTCTCTTCCATCAGAAAGCTTTAC-3′
G-CSF	5′-AGAGAGTGTCCGAGCAGCAC -3′	5′- CAAGTGAGGAAGATCCAGGG-3′
CXCL1	5′- CTTCCTCCTCCCTTCTGGTC -3′	5′- GAAAGCTTGCCTCAATCCTG-3′
CCL2	5′- AGGTGACTGGGGCATTGAT-3′	5′-GCCTCCAGCATGAAAGTCTC-3′

EF1a, Elongation factor 1a.

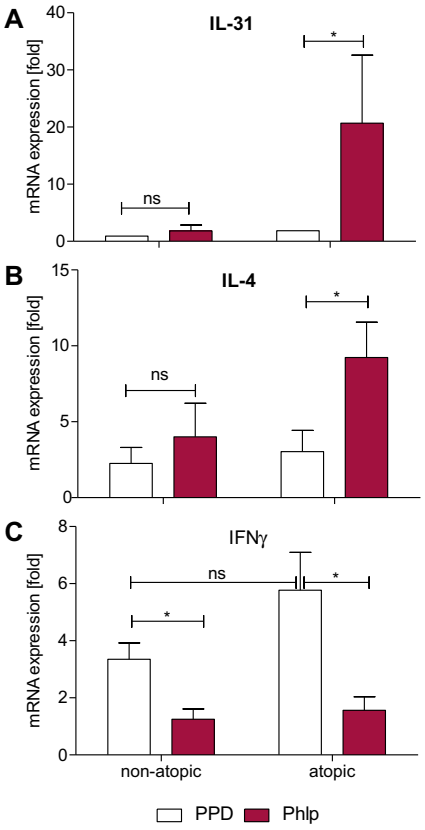

FIG 1. Expression of IL-31 is enhanced in atopic donors. PBMCs from nonatopic donors and donors with grass pollen allergy (n = 6 per group) were stimulated for 7 days with *P pratense* (10 μg/mL) or PPD (1 μg/mL). Quantitative real-time PCR was carried out to measure IL-31 **(A)**, IL-4 **(B)**, and IFN-γ **(C)** levels. Data are normalized to elongation factor 1a housekeeping gene expression and shown relative to the nonatopic polyclonal condition. $*P \leq .05$, as determined by using the paired *t* test. *ns*, Not significant.

response to normalize the PCR results. Although all T cells were stimulated, as opposed to just the antigen-specific portion, the polyclonal stimulation had very low gene expression for all genes measured (data not shown).

T_H2 clones express IL-31

IL-31 has been shown to be upregulated in T-cell cultures polarized to the T_H2 phenotype. $CD4^+$ T-cell clones were generated from peripheral blood from 2 donors with grass pollen allergy to study IL-31 expression on a single-cell level. IL-31 was then quantified by means of ELISA in the supernatants of these cultures. A lack of suitable intracellular fluorescence-activated cell sorting (FACS) antibodies for IL-31 means coexpression of IL-4 and IL-31 has not yet been demonstrated. As shown in Fig 2, *A*, IL-31 was highly expressed by T_H2 clones (mean ± SEM, 517 ± 159.9 pg/mL) and significantly less by T_H1 clones (mean ± SEM, 39.15 ± 17.34 pg/mL). IL-31 was expressed uniquely by IL-4^+ clones, including T_H0 cells. However, there was no correlation between IL-4 and IL-31 expression (see Fig E2 in this article's Online Repository at www.jacionline.org). Two of 3 T_H22 clones also produced IL-31 (mean ± SEM, 283.3 ± 146.6 pg/mL). In parallel with this study, an epigenetic study of T_H1- and T_H2-polarized T-cell cultures shows a difference in histone methylation at the IL-31 gene between phenotypes (Fig 2, *B*). H3K27 trimethylation, a marker of silenced genes,[21] is enriched over the IL-31 gene in chromatin from T_H1 cultures but is absent in T_H2 chromatin. H3K4me1, a marker of potential regulatory domains,[22] is differentially enriched in the 2 cell types, and these sites of enrichment map to cell type–specific DNase1-hypersensitive sites. However, there is only sparse H3K4 trimethylation at the IL-31 transcriptional start site in T_H2 culture, which is a marker of potentially active genes.[23] The IL-31 gene is located within an intron of the LRRC43 gene, and these 2 genes are transcribed from different DNA strands. Both LRRC43 and the neighboring gene B3GNT4 show broadly equivalent enrichment of H3K4me3 at their transcriptional start sites in both T_H1 and T_H2 chromatin indicative of equivalent transcription. These data suggest that T_H1 and T_H2 chromatins are differently organized at the IL-31 gene and that IL-31 is more likely to be expressed from T_H2 than T_H1 cells but might need an additional stimulus to promote this process.

IL-31 is dependent on IL-4

IL-4 is the main polarizing cytokine to drive T_H2 differentiation and is produced specifically by T_H2 cells. We wanted to investigate whether human IL-31 is induced by IL-4 or whether IL-31 is constitutively expressed once a cell is fully differentiated to T_H2. T_H2 clones were restimulated as before but in the presence of blocking/neutralizing antibody to IL-4 (Fig 3, *A*). This significantly reduced IL-31 expression from most of the clones, highlighting the importance of the presence of IL-4 (mean ± SEM expression of anti–IL-4–treated clones, 66.18% ± 9.28% of baseline IL-31 expression, a 1.5-fold decrease). A 1.7-fold decrease (201.7 ± 37.92 pg/mL [± SEM] untreated vs 116.9 ± 31.46 pg/mL anti–IL-4 treated) was also observed for all non-T_H2 clone phenotypes (T_H1, T_H17, T_H22, and T_H0; Fig 3, *B*), highlighting the importance of autologous IL-4 for IL-31 expression. Both memory (CD45RO) and naive (CD45RA)

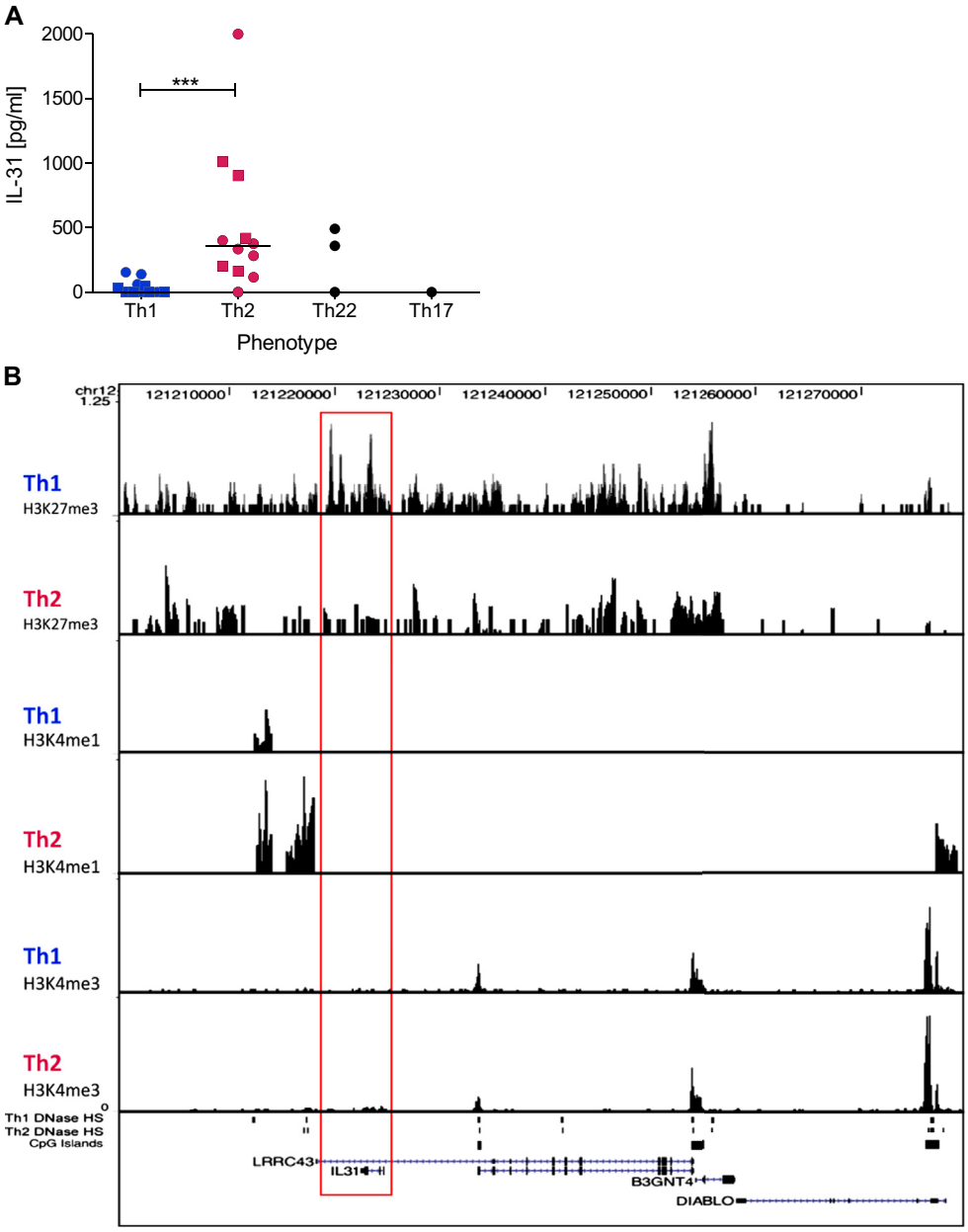

FIG 2. Generation and characterization of T-cell clones. CD4$^+$ T cells from donors with grass pollen allergy (n = 2) were cloned by means of limiting dilution. Different symbols represent different donors. **A,** IL-31 levels measured in supernatants of clones. *Horizontal bar* shows grand mean. ***$P \leq .0005$, as determined by using the unpaired *t* test. **B,** Chromatin architecture of the IL-31 locus. A custom track view (Hg 18 build) is shown of the T_H1- and T_H2-specific DNAase hypersensitivity sites; histone modifications H3K4me1, me3, and H3K27me3 in T_H1 and T_H2 cells; and positions of CpG islands in this region of chromosome 12.

T cells show a dose-dependent expression of IL-31 in response to IL-4 from memory and naive T cells, which varies considerably among different donors (Fig 3, *C* and *D*).

IL-31 can be expressed by T_H1 cells

Following on from the observation that IL-31 expression from T_H2 cells can be induced by autologous IL-4, we washed and re-stimulated T_H1 clones in the presence or absence of IL-4 (Fig 4, *A*). Although levels were very low, in comparison with T_H2, IL-31 expression was significantly increased in the presence of 20 ng/mL IL-4 (mean ± SEM, 47.85 ± 20.19 pg/mL vs 94.97 ± 26.07 pg/mL). We analyzed the IL-4 receptor expression on T-cell clones using flow cytometry and found it to be small but detectable (see Fig E3 in this article's Online Repository at www.jacionline.org) on all clone phenotypes, confirming the T_H1 clones' ability to respond to IL-4. This IL-31 expression by T_H1 clones is only transient, as shown in Fig 4, *B*. When restimulated 2 weeks after IL-4 treatment, IL-31 levels from these clones had returned to those of the untreated clones. Similarly, when T_H1 clones were treated with IL-4, there was no difference in T_H2 or T_H1 cytokine expression, as shown by IL-13 and IFN-γ

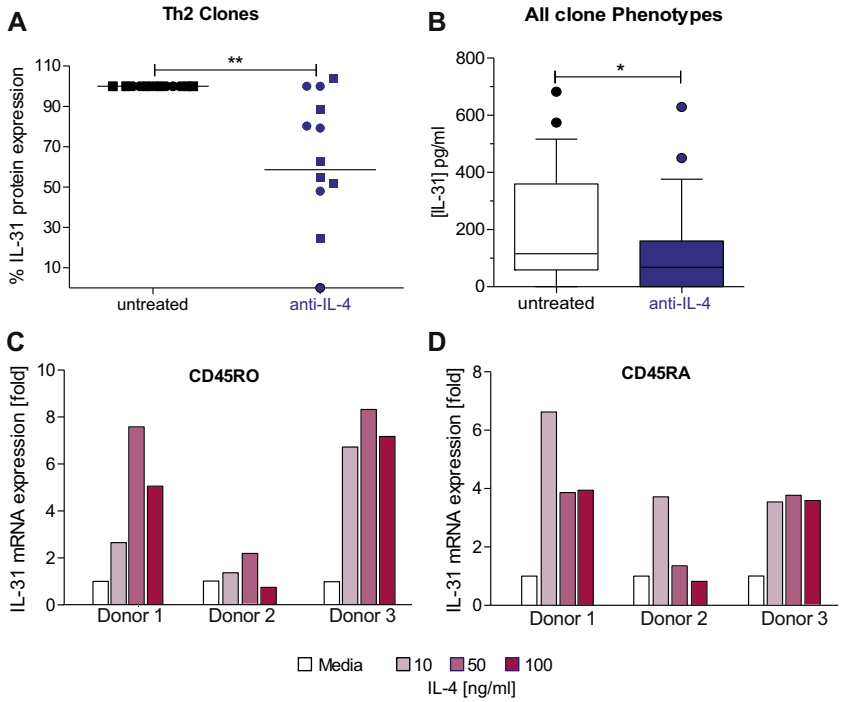

FIG 3. IL-31 expression is dependent on IL-4. Clones were stimulated for 48 hours with or without anti–IL-4 (5 μg/mL). **A** and **B,** IL-31 levels were measured by using ELISA for T_H2 clones (Fig 3, *A*) and all clone phenotypes except T_H2 (Fig 3, *B*). $*P \leq .05$ and $**P \leq .005$, as determined by using the paired *t* test. **C** and **D,** $CD45RO^+$ (Fig 3, *C*) and $CD45RA^+$ (Fig 3, *D*) T cells of 3 genetically independent donors were polyclonally stimulated for 48 hours with increasing concentration of IL-4. IL-31 mRNA was measured by using real-time PCR, and data are normalized to elongation factor 1a and shown relative to the media control.

expression, respectively (Fig 4, *C* and *D*). Although IL-13 concentrations from T_H1 clones appear to be high, they were modest relative to concentrations from T_H2 clones.

IL-31 synergizes with IL-4 and IL-13 in the induction of epithelial genes

The lung epithelium is the point of contact of the body with the environment and as such plays an important role in allergic asthma and rhinitis. As shown in Fig 5, *A* and *B*, primary NHBEs express IL-31 receptor. We screened NHBEs for a number of genes that are often overexpressed in patients with allergic inflammation for a response to treatment with recombinant IL-31 (see Fig E4 in this article's Online Repository at www.jacionline.org). CCL2, also known as monocyte chemoattractant protein 1, showed a nonsignificant response to IL-31 (2.07-fold ± 0.53-fold change [± SEM]; Fig 5, *C*). When combinations of IL-31 and the T_H2 cytokines IL-4 and IL-13 were applied to NHBEs, a synergistic increase in CCL2 expression was observed (IL-13 and IL-31, 17.02 ± 1.51–fold increase vs IL-31 alone [$P \leq .0008$], and IL-4 plus IL-31 showed a 29.54 ± 6.13–fold fold increase vs IL-31 alone [$P \leq .011$]; Fig 5, *C*). Treatment with all 3 cytokines did not induce the highest CCL2 expression, perhaps because of an oversaturation of receptor signaling pathways.

For the genes CXCL1, IL-8, and granulocyte colony-stimulating factor (G-CSF), the pattern was slightly different. IL-31 did not enhance the effect of IL-4 and IL-13 separately but could promote the gene expression induced by IL-13 plus IL-4 by more than the fold change induced by IL-31 alone. IL-31 alone induced 1.7 ± 0.2–fold CXCL1 but added 4.2 ± 1.9–fold to IL-4 plus IL-13–induced CXCL1 expression. Alone, IL-31 induced 1.2 ± 0.2–fold IL-8 expression but added 2.9 ± 2.1–fold to IL-13 plus IL-4–induced IL-8 expression. G-CSF was induced 1.4 ± 0.2–fold by IL-31, but the difference between IL-4 plus IL-13 and IL-4 plus IL-13 plus IL-31 was a 1.6 ± 1.1–fold increase. For these 3 genes, the combination of all 3 cytokines induced the greatest expression (Fig 5, *D* and *F*).

IL-33 can induce IL-31 from T cells

IL-33 is a proinflammatory cytokine that promotes the development of T_H2 cells.[16,24] As shown in Fig 6, IL-33 had a slight influence on IL-31 expression (1.5 ± 0.2–fold for naive cells and 3.9 ± 2.7–fold increase for memory cells [± SEM], both relative to media control) from naive and memory T cells after a 48-hour stimulation. This is less than the effect of IL-4–driven IL-31 expression (4 ± 0.4–fold increase for naive and 6.6 ± 4–fold increase for memory cells [± SEM]). However, when IL-4 and IL-33 were combined, then an increase in IL-31 levels (13 ± 2.6–fold increase for naive and 19.73 ± 13.1–fold increase for memory cells [± SEM]) was observed that was greater than the combined individual effects of IL-4 and IL-33 and, in the case of naive cells, statistically significant. In correlation with Fig 3, *C*, there was no significant difference between the ability of naive and memory T cells to respond to IL-4 and IL-33 to produce IL-31.

DISCUSSION

IL-31 has been shown to be upregulated in patients with allergic disorders, such as atopic dermatitis,[3] but it has been unclear whether allergen stimulation directly induces IL-31 expression or whether a secondary factor, such as bacterial infection or

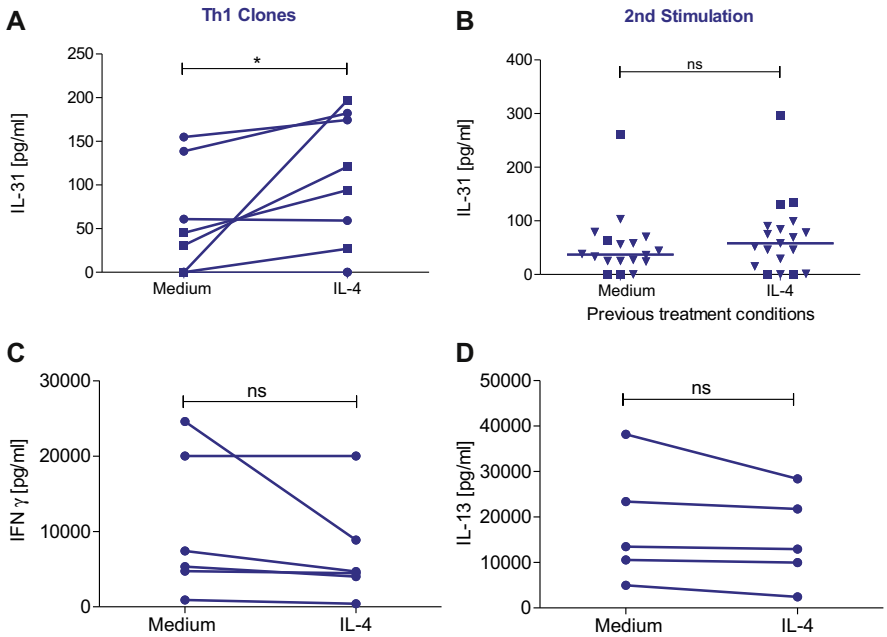

FIG 4. IL-4 can induce IL-31 from T_H1 clones. **A, C,** and **D,** T_H1 clones from 2 donors (donor origin indicated by *round* or *square points*) were restimulated with or without 20 ng/mL IL-4, and IL-31 (Fig 4, *A*), IFN-γ (Fig 4, *C*), and IL-13 (Fig 4, *D*) levels were measured by using ELISA. $*P \leq .05$, as determined by using the paired *t* test. **B,** These clones were then cultured for a further 10 days and polyclonally stimulated as before but without IL-4. IL-31 was measured again by using ELISA. No significant difference was, as determined by using the unpaired *t* test. *ns*, Not significant.

inflammation, is necessary. Our results demonstrate that IL-31 can be induced directly by allergen stimulation. Although it is impossible to determine which cells are producing IL-31 by measuring mRNA expression in PBMC culture, previous literature[1] supports the theory that the majority of these IL-31–producing activated cells are allergen-specific memory T cells. IL-31 expression was increased in atopic donors similar to IL-4 expression,[25] and therefore this result is in line with the previously suggested link between IL-31 and allergy.

Human allergen-specific T cells generally possess a T_H2 phenotype,[20] and therefore we asked whether IL-31 is coexpressed with T_H2 cytokines. We demonstrate that T_H2 clones produced much higher amounts of IL-31 than other subsets, such as T_H1. There was no significant correlation between IL-4 and IL-31 expression when looking at all collected clones, which might have been due to the wide range of IL-4 expression levels observed from the T_H2 clones (underrepresentation of IL-4 intermediate/low clones). The IL-4 high-producing clones might have induced differential IL-4 receptor, oncostatin M receptor, or IL-13RA expression in the long-term cultures, leading to variations in IL-31 expression.

For the first time, the current data show IL-31 production from naturally derived human T_H2 cells as opposed to *in vitro* polarized T_H2 cells. Interestingly, IL-31 was also detected in 2 of 3 T_H22 clones, suggesting IL-31 expression is not limited to T_H2 cells. This view was confirmed by the chromatin methylation status of T_H1 and T_H2 cells. Although the IL-31 gene is in an H3K27me3-enriched environment in T_H1 cells, there was not a peak of permissive H3K4 methylation in the T_H2 cells. Instead of considering IL-31 to be enhanced in T_H2 clones, perhaps a more accurate view is that IL-31 is selectively inhibited in T_H1 clones.

T_H2 clones maintain IL-31 expression independent of any external stimulation or coculture. If IL-31 is not specifically expressed by T_H2 clones, as the T_H22 clones suggested, then could IL-31 expression be dependent on a factor expressed by T_H2 cells? We showed that the elimination of autocrine IL-4 by washing T_H2 clones and neutralizing any *de novo* produced IL-4 substantially reduced IL-31 expression. This result implies that IL-31 requires a constant source of exogenous IL-4 or IL-4 receptor signaling to be expressed. T_H2 cells might always be associated with IL-31 expression because they constantly secrete IL-4[26] into the immediate vicinity of the surface IL-4 receptor but are not epigenetically imprinted toward an inheritable IL-31^+ phenotype. Moreover, there was no difference in response to IL-4 between naive and memory T cells.

Recent analysis of the IL-31 promoter has revealed a conserved signal transducer and activator of transcription 6 binding site,[27] which could link IL-4 signaling[28] with IL-31 transcription. Previous links between IL-31 and T_H2 cells might have been observed because of the high levels of IL-4 present in both polarizing culture conditions and produced by the cells themselves. This observation led us to the new hypothesis that any T cell could produce IL-31 if exposed to IL-4.

When IL-4 was added to T_H1 clones during a 48-hour restimulation, a marginal but significant upregulation of IL-31 was observed. This was an unexpected result because the ChIP results in Fig 2, *C*, suggested the IL-31 gene was likely to be silenced in T_H1. Also, IFN-γ produced by T_H1 clones has been shown to downregulate IL-4 receptor expression.[29] These data demonstrate that IL-31 is not a T_H2 cytokine but rather an IL-4–responsive gene. We showed that this effect is temporary and that the IL-31 expression from cells treated with IL-4 returns to baseline levels on withdrawal of IL-4. Although T_H1 cells are responding to IL-4, they are fully differentiated to T_H1 without exhibiting plasticity and diverging toward a T_H2 phenotype, as demonstrated

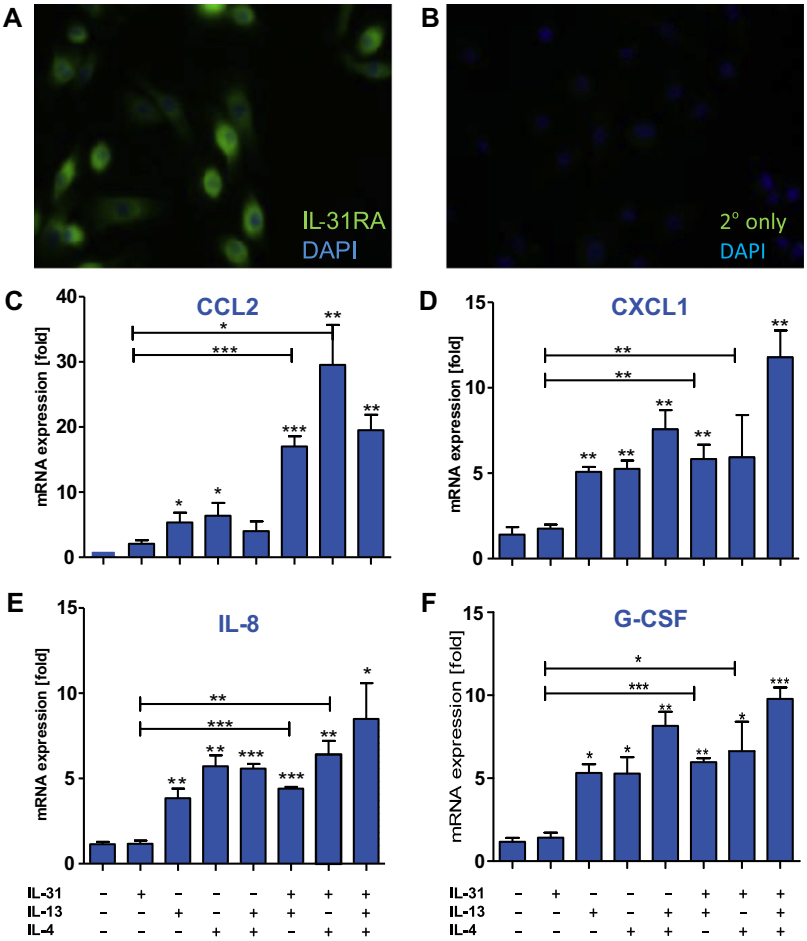

FIG 5. NHBEs can respond to IL-31. **A** and **B,** NHBEs were stained with anti–IL-31RA and Alexa Fluor 488 (*green*; Fig 5, *A*) or Alexa Fluor 488 only (Fig 5, *B*). Nuclei were counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride (*DAPI*; *blue*). Seventy percent confluent NHBEs were stimulated for 8 hours with combinations of IL-4, IL-13, and IL-31 at 20 ng/mL, 20 ng/mL, and 50 ng/mL, respectively. **C-F,** RT-PCR was used to measure CCL2 (Fig 5, *C*), CXCL1 (Fig 5, *D*), IL-8 (Fig 5, *E*), and G-CSF (Fig 5, *F*) levels. Data shown are relative to the 18s housekeeping and relative to media control. $^{*}P \leq .05$, $^{**}P < .01$, and $^{***}P \leq .0005$, as determined by using the paired *t* test.

by the lack of effect of IL-4 on either promoting IL-13 or inhibiting IFN-γ expression. Similarly, neutralizing IL-4 from differentiated T_H2 clones does not affect the expression of IL-13 (observation in house). This would suggest that IL-31 expression does not result from a T_H1 to T_H2 conversion but is upregulated as a response to exogenous IL-4. The IL-4 receptor is not restricted to T cells, and work is ongoing to explore whether any cell that responds to IL-4 can also produce IL-31. This might lead to IL-31 having a much broader role than appreciated currently, with greater clinical effects to be gained through pharmaceutical neutralization.

Functional IL-31 receptor expression has been described on keratinocytes and dorsal ganglions. We are interested in the lung epithelium as a point of contact with airborne allergens and locality of allergen-induced inflammation in patients with allergic asthma and hay fever. IL-31RA expression was confirmed by means of immunohistochemistry on NHBE cultures. The present data confirm, by using primary human cells, the results of studies that have used the bronchial cell line BEAS-2B.[10] Treating NHBEs with increasing concentrations of IL-31 did not reveal significant gene expression changes for genes commonly overexpressed in patients with allergic inflammation, such as chemokines, cytokines, and growth factors. In contrast, epithelial cells treated with combinations of IL-4, IL-31, and IL-13 showed significantly increased gene expression of IL-8 and CCL2, both substances with potent proinflammatory actions that are overexpressed in patients with allergic asthma.[30-32] This combinatory threshold for gene induction is also observed for other cytokines that are recognized by structural cells.[33,34] Although we did not find IL-31 to influence the expression of genes that are strongly associated with allergy, such as thymus and activation-regulated chemokine (CCL17) and thymic stromal lymphopoietin, our evidence suggests a role for IL-31 as a cytokine that amplifies the effects of T_H2 cytokines and promotes neutrophilic inflammation also by induction of G-CSF. Thus IL-31 might represent a target for neutralization in allergy and asthma therapy, which would be tissue specific and avoid the side effects of neutralizing a more pleiotropic and systemically acting cytokine.

A gene found to be induced from NHBEs by IL-31 and not IL-4 or IL-13 was IL-33. This is a newly described cytokine that is thought to be very important in promoting T_H2-driven inflammation, acting directly on T cells to polarize them toward T_H2 and

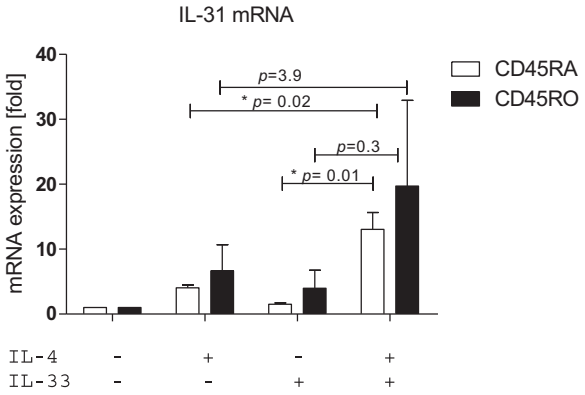

FIG 6. IL-33 can induce IL-31 from T cells. CD45RO$^+$ and CD45RA$^+$ T cells from donors with grass pollen allergy (n = 3) were polyclonally stimulated for 48 hours with IL-4 (20 ng/mL) and IL-33 (20 ng/mL). RT-PCR was performed to measure IL-31 levels. Data shown are normalized to elongation factor 1a and shown relative to the media control. A paired *t* test was used to determine significance.

acting as a chemoattractant for T_H2 cells.[24] Our study showed that IL-33 could induce IL-31 from T cells. This result is relevant because it shows that the epithelium can directly drive IL-31 expression independent of IL-4. However, this result also shows that IL-31 is perhaps not as separate from T_H2 differentiation mechanisms as previously described. Further studies on the effect of IL-33 on other cells will help demonstrate whether IL-31 is generally an IL-4 and IL-33 response gene.

To summarize, IL-31 appears to be strongly associated with allergy and induced by allergen stimulation, with an enhanced expression originating from T cells of donors with grass pollen allergy. IL-4 receptor signaling is essential to IL-31 expression, with IL-31 levels significantly reduced within 48 hours of IL-4 neutralization, even from non-T_H2 clones.

IL-31 has a minor effect on NHBE gene expression alone but seems able to synergize with the T_H2 cytokines IL-4 and IL-13 to promote gene expression from NHBEs, suggesting a proinflammatory role and a perhaps a role of amplifying existing T_H2 reactions. In turn, tissue-derived IL-33 is able to act with IL-4 to induce IL-31 expression from T cells. These data would suggest that IL-31 plays a crucial role amplifying and maintaining T_H2-driven inflammation, such as that observed in patients with atopic dermatitis, allergic asthma, and inflammatory bowel disease.

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Key messages

- **IL-4 can induce IL-31 from TH1 and TH2 T-cell clones.**
- **IL-31 levels are increased in PBMCs from patients with allergic rhinitis and promotes T_H2-driven lung inflammation.**
- **IL-31 is induced by IL-4 and IL-33 and helps induce inflammatory chemokines from bronchial epithelium.**

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METHODS

PBMC isolation

Heparinized blood was processed within 2 hours of bleeding. Blood was diluted 1:1 with PBS before being layered on FicollPaque plus (Pharmacia, Uppsala, Sweden), and PBMCs were isolated by means of density gradient centrifugation. PBMCs were cultured at 2×10^6/mL in 24-well plates in culture medium: RPMI 1640 supplemented with 5% human AB serum, 5 mmol/L glutamine, and 1× penicillin and streptomycin solution (all from Invitrogen, except serum from Sigma-Aldrich). Stimulation was through addition of whole Phl p 5 protein extract (ALK-Abelló), the allergen component of timothy grass, *P pratense* pollen at 10 μg/mL, PPD (Statens Serum Institute, Copenhagen, Denmark) at 1 μg/mL, or polyclonal stimulation with plate-bound anti-CD3 and anti-CD28 (both from R&D Systems).

$CD4^+$ naive and memory T-cell isolation

$CD4^+$ T cells were isolated by means of negative selection with magnetic beads conjugated to antibodies for CD8, CD14, CD16, CD19, CD36, CD56, CD123, T-cell receptor γ/δ, and CD235a (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocols. The purity, as checked by using FACS, was always 95% or greater. To isolate memory and naive T cells, CD45RO microbeads (Miltenyi Biotec) were used to sort the $CD4^+$ T cells. $CD45RO^+$ T cells were considered memory cells, and $CD45RO^-$ T cells were considered naive cells; again, purity by using FACS was always 93% or greater. T cells were cultured in culture medium. T cells were stimulated with plate-bound anti-CD3/anti-CD28, and as before, cultures of longer than 48 hours were also supplemented with IL-2 (Peprotech, Rocky Hill, NJ) at 20 ng/mL.

ELISA

Clones were seeded at 200,000 cells per well of a 96-well flat-bottomed plate in 200 μL of cloning media and stimulated with plate-bound anti-CD3 and anti-CD28. Forty-eight hours later, cell-free cell-culture supernatants were removed and assayed for IL-31, IL-4, IFN-γ, IL-22, and IL-13. All ELISA kits were used per the manufacturer's recommended protocol and were all Duoset kits from R&D Systems, except IL-31, which was from Peprotech.

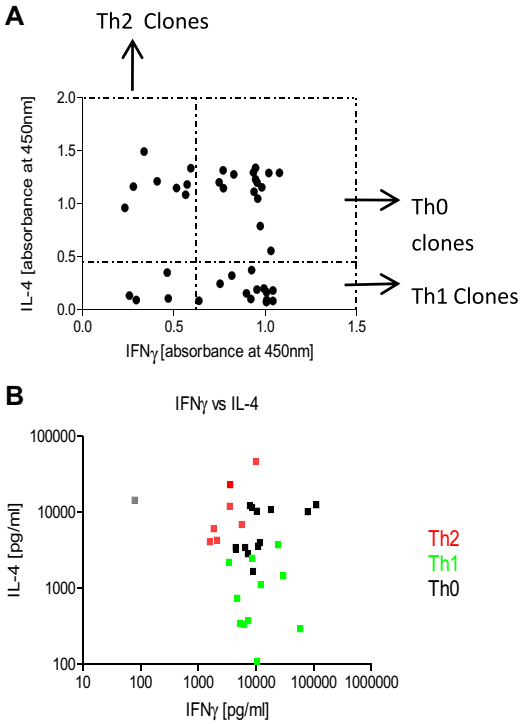

FIG E1. Characterization of clones. T-cell clones were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours. Supernatants were analyzed by means of ELISA for IL-4 and IFN-γ. **A,** Absorbance values for each ELISA for each clone. **B,** Actual concentration values for these characterized clones.

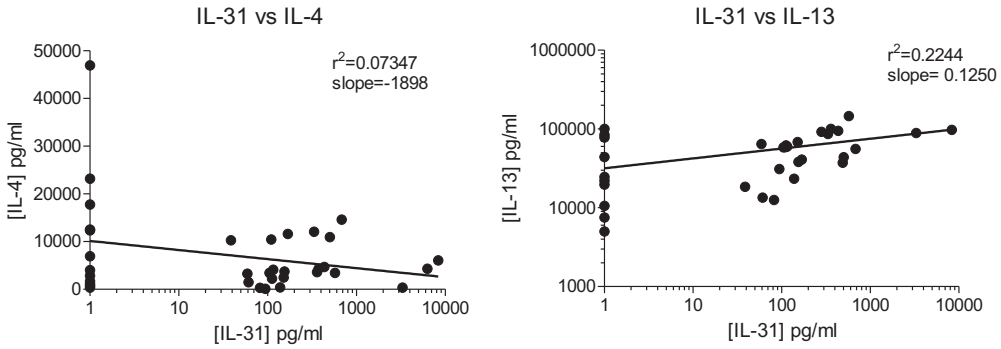

FIG E2. Expression of cytokines by T-cell clones versus IL-31 expression. Clones were stimulated for 48 hours with plate-bound anti-CD3 and anti-CD28. Supernatants were analyzed by using ELISA. Any correlation was determined by means of nonlinear regression.

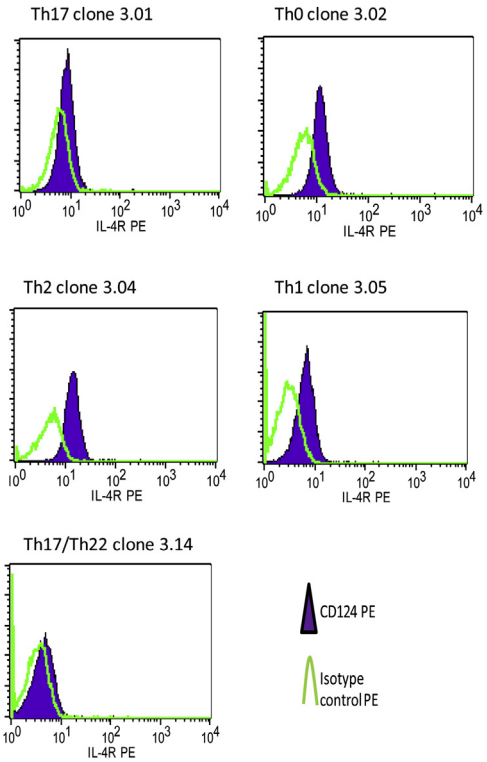

FIG E3. IL-4 receptor (CD124) expression on different T-cell clone phenotypes. Resting T-cell clones were stained with either CD124 phycoerythrin *(PE)* or PE isotype matched control before being analyzed by using flow cytometry. Histograms show fluorescence intensity.

Gene detected	Fold change following cytokine treatment relative to media control		
	IL-31	IL-4	IL-13
CCL2	12.35	15.37	16.7
CCL5	0.82	0.67	2.2
CCL11	0.33	0.38	0.28
CCL17	0.44	0.65	0.52
CCL22	0.64	0.49	0.47
CXCL1	0.93	1.66	1.2
EGF	1.15	0.29	0.52
G-CSF	2.16	2.15	2.86
GM-CSF	1.67	0.69	0.98
IL-6	0.9	0.24	0.52
IL-8	1.3	1.96	1.96
IL-25 sv2	0.47	0.49	0.37
IL-33	1.54	0.33	0.6
IL-31RA	0.73	1.12	0.69
Muc5AC	2.11	0.83	1.13
TGFβ	1.06	0.7	0.44
TSLP	0.46	0.96	-

FIG E4. Panel of genes analyzed during preliminary studies of the effect of IL-31 on NHBEs. Seventy percent confluent NHBEs were treated with IL-31, IL-13, or IL-4 (all 50 ng/mL) for 8 hours. RT-PCR was performed, and data are normalized against 18s housekeeping expression. Values show fold change relative to media control: *blue* indicates downregulation, and *orange* indicates upregulation (n = 2 for each condition).