Original article

Eosinophilic inflammation: mechanisms regulating IL-5 transcription in human T lymphocytes

Background: Interleukin (IL)-5 is a key regulator of eosinophilia in allergic inflammation and parasite infections but the mechanisms regulating IL-5 expression in activated human T lymphocytes are poorly understood. From studies on mouse cells, the activation protein (AP)-1 and GATA-3 sites in the proximal promoter region appear to be important in IL-5 regulation but the significance of an adjacent Ets/nuclear factor of activated T cell (NFAT) site has been less clear.

Methods: Interleukin-5 transcriptional activity was measured by transfection of reporter genes into the human HSB-2 cells and normal T lymphocytes. Expression vectors encoding transcription factors were used for transactivation studies and IL-5 expression measured using reporter genes and mRNA levels. Transcription factor binding was shown with chromatin immunoprecipitation (ChIP).

Results: HSB-2 cells showed high inducible expression of IL-5 mRNA. Mutation of reporter gene plasmids showed the Ets/NFAT site was of equal importance to the AP-1 and GATA-3 sites in regulating IL-5 transcription. Transactivation by Ets1 increased luciferase expression 15-fold, in the absence of stimulation, and AP-1 (c-Fos/c-Jun) and GATA-3 gave transactivations of 85-fold, and 100-fold, respectively. Synergistic interactions were demonstrated between Ets1, GATA-3 and AP-1. Dominant-negative AP-1 inhibited IL-5 transcription. Transactivation by GATA-3 and synergy between GATA-3, Ets1 and AP-1 were verified measuring IL-5 mRNA levels. Chromatin immunoprecipitation showed increased binding of Ets1 and GATA-3 to the IL-5 promoter after stimulation. The importance of the Ets1 site and of synergistic interactions between the three transcription factors were verified with primary human T cells.

Conclusion: Ets1, GATA-3 and AP-1 synergize to regulate IL-5 transcription in human T cells.

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Interleukin (IL)-5 is one of the key cytokines produced by activated T lymphocytes which regulate immune and inflammatory responses. Interleukin-5 appears to play a central role in the regulation of eosinophilia and contributes to several human diseases, including asthma (1–3). The importance of IL-5 in eosinophilic inflammation (4, 5) makes it desirable to obtain a better understanding of the mechanisms regulating IL-5 transcription in human T cells.

Most previous studies of IL-5 expression have been with mouse cells. T-cell receptor stimulation activates protein kinase C (PKC) and increases intracellular calcium. This is also achieved by phorbol 12-myristate

13-acetate (PMA) and ionophore but optimal induction of IL-5 expression requires PMA and cAMP, suggesting that both PKC and protein kinase A activation is involved (6, 7). Both mouse and human IL-5 expression require protein synthesis (8, 9).

Homology in the flanking regions of the mouse and human IL-5 genes is limited to the proximal promoter region from –80 to –20. Conserved lymphokine element 0 (CLE0), located at –53 to –39 is involved in positive regulation of the mouse IL-5 gene (10, 11). It consists of putative activation protein (AP)-1 and Ets/nuclear factor of activated T cell (NFAT) elements in which the AP-1 site binds JunB and JunD in D10 cells (12); c-Fos, JunB and JunD in EL-4 cells (13) and c-Fos, JunB and NFAT in EL-4 cells (14). In contrast, the putative Ets/NFAT site has been much less studied and only recently has evidence supporting a role for Ets1 in mouse IL-5 transcription

Abbreviations: AP-1, activation protein 1; NFAT, nuclear factor of activated T cells; PMA, phorbol 12-myristate 13-acetate; cAMP, cyclic adenosine monophosphate; CsA, cyclosporine A.

been obtained (15). The GATA site (–71 to –66) located upstream of the CLE0 element is important for mouse IL-5 transcription (16–18). GATA-3 is specifically expressed in mouse Th2 cells and is important in the expression of Th2-specific cytokines (18). There have been contradictory findings on the elements in the proximal promoter region in human IL-5 regulation. Support for involvement of CLE0 was obtained by Yamagata et al. (19) and Thomas et al. (20) but not by Stranick et al. (21). Ets protein could interact with the viral transactivator Tax to stimulate IL-5 transcription in Jurkat cells (22). In terms of a role for GATA, deletion data from Gruart-Gouilluex et al. (23) indicated no involvement of the proximal GATA site in promoting human IL-5 transcription, whereas a selective role for GATA-4 rather than GATA-3 at this site was shown with ATL-16T cells (24). Two studies using T lymphocytes from asthmatic patients have shown a role for the proximal promoter region to –119 in human IL-5 transcription (25, 26) but detailed transactivation studies with transcription factors have not been carried out with primary T cells.

Studies of human IL-5 expression have suffered because of lack of suitable human T-cell lines. Some studies have utilized mouse T-cell lines, the human leukaemia ATL-16T which expresses IL-5 constitutively or Jurkat cells which do not express IL-5. More recently the PER-117 human T-cell leukaemia line has been used (27) but this cell line has restricted availability.

In the present study, we demonstrate the utility of the human T-cell leukaemia HSB-2 as a model for IL-5 regulation studies. Interleukin-5 expression in this cell line is similar to that in normal human T cells (28, 29). We have studied the importance of the Ets/NFAT site in the proximal promoter region of the human IL-5 gene in comparison with the adjacent AP-1 and GATA sites using HSB-2 cells and looked for synergistic interactions between the transcription factors binding at these sites. We found that the Ets/NFAT site in the proximal promoter region was obligatory for IL-5 expression in HSB-2 cells as were the AP-1 and GATA sites. In addition, Ets1, AP-1 (c-Fos/c-Jun) and GATA-3 showed strong transactivations and synergistic interactions which was dependent on the integrity of each of the three transcription factor-binding sites. The importance of the Ets1 site and the synergy between the three transcription factors was verified using primary human T cells. The results suggest that a higher order complex analogous to the enhanceosome of the interferon- β promoter (30) could be involved in regulating human IL-5 transcription in T cells.

Methods

Cell culture and isolation of human T cells

Human HSB-2 T cells were maintained in RPMI-1640 medium (28). For primary human T cells, fresh blood was collected from healthy

volunteers (procedures monitored by the Human Research Ethics Committee, Australian National University), mononuclear cells prepared (25, 31) and T cells isolated using RosetteSep™ Kit (Stem Cell Technologies, Vancouver, Canada). The human T cells were cultured with 2 μ g/ml of phytohemagglutinin (PHA) for 24 h, washed once and resuspended in medium containing human IL-2 and cultured for 6–7 days. More than 99% of the cells were CD3-positive by FACS.

Plasmid construction

The -1200hIL-5Luc reporter contains 1200 bp preceding the transcription initiation site of the human IL-5 promoter in pXP1. The mutations of AP-1-, Ets/NFAT- and GATA-binding sites were made using Quick Change™ (Stratagene, La Jolla, CA, USA) and verified by sequencing. The expression plasmids for GATA-3, GATA-4, c-Jun, c-Fos, Ets1 and E1f1, NFATp and NFATc (15) and dominant-negative c-Jun (A-Jun) and c-Fos (A-Fos) (32) were previously described.

Transfection assays

About 5×10^6 cells were incubated with 20 μ g of DNA (5 μ g IL-5 reporter plasmids, 5 μ g expression plasmids and empty plasmid to a total of 20 μ g) for 15 min at room temperature, and subjected to electroporation at 280 V and 975 μ F capacitance using a GenePulser (Bio-Rad, Hercules, CA, USA). The cells were incubated for 5 ml of medium at 37°C for 20 h, then treated with or without PMA (25 ng/ml), cAMP (1 mM) and calcium ionophore A23187 (1 μ M) for 9 h. The method used for transactivation effects on endogenous IL-5 mRNA was described previously (15).

Transfection of human primary T cells was performed using a Human T cell Nucleofector™ Kit and a Nucleofector™ (amaxa DmbH, Cologn, Germany) according to the manufacturer's instructions. Transfection efficiency was 60% determined by FACS using a GFP expression plasmid. About 3×10^6 cells were mixed with 2 μ g of reporter construct and different expression plasmids in 100 μ l of Human T cell Nucleofector™. After transfection, cells were stimulated with PMA/cAMP/Ionophore as described above.

Luciferase assays

For HSB-2 cells, luciferase assays were carried out using a Top-Counter (Packard, Ramsey, MN, USA) as described previously (15). The linearity of the assay was confirmed for high activity samples. The background was very low and subtracted from all values. Mean and standard deviations of at least three independent experiments are shown in the figures. The data were subjected to statistical analysis using unpaired *t*-tests, with resultant *P*-values of <0.05 considered significant. The data for transactivation and synergies discussed in the text had *P*-values of <0.004 and were highly significant. The effects of GATA-3 and GATA-4 were not significantly different.

For human primary T cells, luciferase activity was determined using a Dual-Glo™ Luciferase Assay System (Promega, Madison, WI, USA).

Real-time PCR analysis

The IL-5 mRNA analyses were performed as described (15) with primers (forward: 5'-ACTCTGCTGATAGCCAATGAG-3',

reverse: 5'-TCCAGTGTGCCTATTCCCTGA-3') and ubiquitin-conjugating enzyme E2D as control.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) assays were carried out as described (15) with Ets1 and GATA-3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and primer set across the IL-5 proximal promoter region (forward: 5'-CATTGGAAA-CATTAGTTTCACG-3', reverse: 5'-TGCGTCCCCAGTCAA-TTTAT-3').

Results

Use of HSB-2 cells to study IL-5 regulation

Interleukin-5 expression in the human T-cell leukaemia HSB-2 is closely similar to normal T lymphocytes. To develop a sensitive reporter gene assay for IL-5 transcription in HSB-2 cells, both transfection efficiency and stimulation were optimized. A plasmid-encoding GFP showed that optimum transfection was achieved with 280 V at 975 μ F where 46% of the cells were viable and 15% of the cells expressed GFP (data not shown).

About 1200 bp of upstream sequence of the human IL-5 promoter plasmid was linked to the luciferase reporter gene (-1200hIL5Luc) and used for assays. Transfected cells were stimulated with PMA, cAMP and calcium ionophore (A23187), individually and in combination. Pairwise combinations of these agents gave up to threefold induction (data not shown), whereas PMA, cAMP and ionophore together induced expression of human IL-5 promoter sixfold in HSB-2 cells (Fig. 1C). The peak of luciferase activity occurred between 9 and 12 h (data not shown). Optimal stimu-

lation of HSB-2 cells resulted in a 1400-fold increase in IL-5 mRNA levels measured by real-time polymerase chain reaction (PCR; Fig. 1B). The sequence of the proximal human IL-5 promoter region shows adjacent putative Ets/NFAT (-40 to -47) and AP-1 (-48 to -55) sites analogous to the mouse CLE0-binding element and an upstream GATA regulatory element (-67 to -72; Fig. 1A). Mutation of each site individually resulted in little change in the basal level of expression in HSB-2 cells but resulted in almost abolition of inducible IL-5 expression (Fig. 1B) indicating that the Ets/NFAT site is of equal importance to the AP-1- and GATA-binding sites.

Use of transactivation to determine the activity of transcription factors in human IL-5 gene transcription

Expression plasmids encoding relevant transcription factors were used to elevate transcription factor levels. Ets1 overexpression resulted in an increase of 15-fold in transcription of the IL-5 promoter in the absence of stimulation (Fig. 2). Elf1 is one of the Ets family of transcription factors which has been shown to be involved in granulocyte-macrophage colony-stimulating factor (GM-CSF) gene expression (33) and NFAT has also been shown to be important in the regulation of a number of cytokine genes, including GM-CSF. Elf1, NFATp and NFATc showed low transactivation of the IL-5 promoter compared with Ets1 (Fig. 2).

AP-1 (c-Fos/c-Jun) and GATA-3 transactivation gave stimulations of 85-fold, and 100-fold, respectively. GATA-4 was also effective. Activation protein-1 and GATA-3 levels are clearly limiting IL-5 expression in HSB-2 cells. Elevation of AP-1 levels occurs

Figure 1. The effects of mutation of the putative activation protein (AP)-1, Ets/nuclear factor of activated T cell (NFAT) and GATA sites of the human interleukin (IL)-5 promoter. (A) The proximal promoter region showing the AP-1, Ets/NFAT and GATA sites and the mutations AP-1-m, Ets-m and GATA-m, respectively. (B) Effect of phorbol 12-myristate 13-acetate/cAMP/Ionophore (P/C/I) stimulation on induction of IL-5 mRNA levels in HSB-2 cells. (C) HSB-2 cells were transfected with the indicated reporter gene plasmids and treated with P/C/I. Luciferase activity is presented as mean and SD (error bars). US, unstimulated.

Figure 2. Transactivation of the human interleukin-5 promoter. The -1200hIL5Luc construct was cotransfected with various expression constructs encoding relevant transcription factors into HSB-2 cells and luciferase activity measured without stimulation. Luciferase activity was calculated relative to reporter alone.

Figure 3. Effect of stimulation on transactivation of the human interleukin-5 promoter. HSB-2 cells were cotransfected with the -1200hIL5Luc construct and expression vectors encoding different transcription factors: (A) Activation protein-1, GATA-3 and GATA-4; (B) Ets1, E1f1, NFATc and NFATp. Transfected cells were stimulated as indicated in the figure. Luciferase activity was calculated relative to reporter alone.

following T-cell activation and may therefore be an important aspect of normal IL-5 induction. This may explain the absolute requirement for protein synthesis for IL-5 expression (9, 34). DNA-binding studies have shown that AP-1 binding to CLE0 is strongly induced by PMA/cAMP stimulation of mouse T-cell lines (12–14).

The effect of stimulation on transactivation was also tested. In each case, stimulation with PMA/cAMP/Ionophore together showed the greatest effect

(Fig. 3). With Ets1 and AP-1 transactivation, stimulation achieved further increases of nine- and 13-fold, respectively, over unstimulated values, whereas with GATA-3 stimulation resulted in a dramatic increase of 72-fold. GATA-3 activation may be particularly effective at stimulating IL-5 transcription. GATA-4 transactivation was also strongly increased by stimulation. Our results do not support the selective involvement of GATA-4 in transcription of the human IL-5 gene previously observed with ATL-16T cells (19).

Synergistic interactions between Ets1, AP-1 and GATA-3

As the binding sites for Ets, AP-1 and GATA are close together in the proximal region of IL-5 promoter with the Ets and AP-1 sites being adjacent, it was of interest to test for cooperative interactions. In the absence of stimulation, Ets1 showed synergistic transactivation of 400-fold with GATA-3 (additive stimulations 115-fold), and 2800-fold with GATA-3 plus AP-1 (additive stimulations 200-fold; Fig. 4A). GATA-3 showed 1100-fold stimulation with AP-1 (additive stimulations 185-fold). GATA-4 was slightly less active than GATA-3 in synergizing with Ets1 and AP-1 and no additional stimulation was achieved with both GATA-3 and GATA-4 present together (Fig. 4A). The significant synergism between the three transcription factors suggests the possibility of a higher order transcriptional complex involving cooperative interactions. p300 has been shown to be involved in the transcription of several cytokine genes and it was of interest to see if p300 could cooperate with the three transcription factors to activate IL-5 transcription. p300 was cotransfected with each of these transcription factors or combinations of them into HSB-2 cells. No evidence of stimulation by p300 was obtained (data not shown).

In addition to reporter gene measurements, IL-5 mRNA levels were measured using real-time PCR. Transactivation by GATA-3 was demonstrated together with synergistic interactions between GATA-3 and Ets1 and GATA-3, Ets1 and AP-1 (Fig. 4D).

Requirement for transcription factor-binding sites

In view of the high level of transactivation of the human IL-5 promoter achieved in the absence of stimulation, it was of interest to determine if the transactivation was dependent on the three transcription factor-binding sites shown to be essential for normal inducible IL-5 gene expression (Fig. 1). The transactivation activity of Ets1 was decreased from 15- to threefold by mutation of the Ets site, and mutation of the GATA-3 site reduced GATA-3 transactivation from 100- to 9.5-fold. Similarly mutation of the AP-1 site reduced the AP-1 transactivation from 85- to

Figure 4. Synergism between transcription factors in the transactivation of the human interleukin (IL)-5 promoter in HSB-2 cells. (A) The -1200hIL5Luc construct was cotransfected with a combination of various indicated expression plasmids into HSB-2 cells. (B) Effect of mutation of the transcription factor-binding sites in the human IL-5 promoter on transactivation in HSB-2 cells. (C) Role of dominant-negative activation protein (AP)-1 on IL-5 transcription. (D) Effect of AP-1/Ets1/GATA-3 transactivation on mRNA levels of IL-5 in HSB-2 cells determined by real-time polymerase chain reaction. In parts (A) and (B) luciferase activity was calculated relative to reporter alone.

5.6-fold (Fig. 4B). These results indicated that the transactivations observed were dependent on the specific transcription factor-binding sites in the proximal promoter region. Significantly, transactivation by each of the three transcription factors was dependent not only in the presence of its own binding site, but also in the binding sites for the other two transcription factors (Fig. 4B). Similarly, the synergistic transactivation of 2800-fold produced by overexpression of AP-1, Ets1 and GATA-3 together in unstimulated cells transfected with -1200hIL5Luc was greatly reduced by mutation of each of the transcription factor-binding sites (Fig. 4B).

Effect of dominant-negative AP-1

The above results suggest that AP-1 plays an important role in regulating human IL-5 transcription in HSB-2 cells and that c-Fos/c-Jun is very effective in transactivation. Experiments were carried out to see if induction of IL-5 expression by PMA/cAMP/Ionophore could be inhibited by dominant-negative AP-1. The repression of IL-5 promoter activity by DN-AP-1 was very significant, and was dose-dependent, reducing the expression to unstimulated levels at a dose of 10 μ g (Fig. 4C) confirming a role for AP-1 (c-Fos/c-Jun) in human IL-5 gene induction.

Figure 5. Binding of Ets1 and GATA-3 to the interleukin-5 promoter in HSB-2 cells *in vivo*. Chromatin immunoprecipitation assays were performed using specified antibodies and a primer set flanking the GATA-3- and Ets1-binding sites.

Measurement of Ets1 and GATA-3 binding using chromatin immunoprecipitation

Chromatin immunoprecipitation assays demonstrated that the binding of Ets1 and GATA-3 to the proximal region of the IL-5 promoter was increased after stimulation of IL-5 expression in HSB-2 cells (Fig. 5). This data strongly supports the role of Ets1 and GATA-3 in regulating human IL-5 expression. Our previous studies on mouse IL-5 transcription also showed specific binding of Ets1 to the mouse promoter using a ChIP assay (15).

Figure 6. Role of the Ets1 site and synergy between Ets1, activation protein-1 and GATA-3 in interleukin (IL)-5 transcription in primary human T cells. Two preparations of human T lymphocytes were used which were transfected with reporter genes and/or expression plasmids for transcription factors. Effect of mutation of the Ets1 site on inducible IL-5 transcription (A and C). Transactivation of IL-5 transcription without stimulation (B and D). In parts (B) and (D) luciferase activity was calculated relative to reporter alone.

Role of Ets1 in synergy with AP-1 and GATA-3 in IL-5 transcription in human primary T cells

Two separate high purity preparations of human T lymphocytes were prepared from healthy human volunteers. The use of a high efficiency transfection procedure with reporter genes allowed inducible IL-5 transcription to be measured. PMA/cAMP/Ionophore stimulation increased IL-5 expression from seven- to 12-fold. Mutation of the Ets1 site reduced IL-5 transcription to unstimulated levels confirming its importance in IL-5 transcription in primary human T cells (Fig. 6A,C). In transactivation experiments without stimulation, synergy between AP-1 and GATA-3, AP-1 and Ets1 and AP-1 with both Ets1 and GATA-3 was demonstrated. Significantly, mutation of the Ets1 site abolished the synergistic stimulation by the combination of the three factors (Fig. 6B,D).

Discussion

The major goal of this study was to investigate the transcription factors regulating IL-5 transcription in the

human T-cell line HSB-2 and in primary T cells. There is a shortage of human T-cell lines showing inducible expression of the IL-5 gene which are suitable for expression assays. The present study showed that the human HSB-2 T-cell leukaemia shows tightly regulated inducible expression of IL-5 mRNA. The sensitive luciferase reporter gene was used to establish a transient transfection system in HSB-2 cells. This system provides a useful model for studying human IL-5 gene regulation and has allowed detailed transactivation and mutational studies to be carried out in the present study.

Earlier work with T lymphocytes from asthmatic patients (25, 26) showed a role for the proximal promoter region in regulating IL-5 transcription but no detailed studies were carried out. We have used HSB-2 cells to look in detail at the role of three putative sites in this region. We found that inducible expression of the human IL-5 gene is severely impaired by mutation of the putative binding sites for Ets/NFAT, AP-1 and GATA-3 in the proximal region of human IL-5 promoter, indicating that the Ets/NFAT site is equally as important as the AP-1 and GATA sites. Our transactivation studies showed that Ets1 is the transcription factor likely to bind at the Ets/NFAT site as it was by far the most active of the Ets and NFAT family members tested giving a 15-fold stimulation in the absence of stimulation (Fig. 2). Activation protein-1 (c-Fos/c-Jun) and GATA-3 also showed strong transactivation and the three transcription factors showed evidence of cooperativity in the synergies observed. Verification of the transactivation effects and synergies was also obtained measuring IL-5 mRNA levels by real-time PCR. Importantly, evidence for increased binding of GATA-3 and Ets1 to the IL-5 promoter after stimulation was obtained using ChIP assays. The role of AP-1 was verified using dominant-negative constructs. An interesting feature of the transactivations and synergistic interactions is that they were all dependent on the integrity of each of the three transcription factor-binding sites. The importance of the Ets1 site and the synergistic interactions with GATA-3 and AP-1 were also demonstrated for the first time with freshly prepared normal human T cells. Our use of a high efficiency transfection method allowed these studies to be done with primary T cells from healthy donors rather than needing to use T cells from asthma patients (25, 26). Taken together our findings suggest that Ets1, AP-1 and GATA-3 may be part of a higher order transcriptional complex involved in regulating human IL-5 expression in T cells. Evidence for interaction between AP-1 and Ets1 in T cells has previously been reported (35). Although the coactivator p300 is involved in regulating a number of cytokine genes, it does not appear to be involved in human IL-5 transcription.

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