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IL-3 synergizes with basophil-derived IL-4 and IL-13 to promote the alternative activation of human monocytes

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Abstract

Basophil-derived IL-4 is involved in the alternative activation of mouse monocytes, as recently shown *in vivo*. Whether this applies to human basophils and monocytes has not been established yet. Here, we sought to characterize the interaction between basophils and monocytes and identify the molecular determinants. A basophil-monocyte co-culture model revealed that IL-3 and basophil-derived IL-4 and IL-13 induced monocyte production of CCL17, a marker of alternative activation. Critically, IL-3 and IL-4 acted directly on monocytes to induce CCL17 production through histone H3 acetylation, but did not increase the recruitment of STAT5 or STAT6. Although freshly isolated monocytes did not express the IL-3 receptor α chain (CD123), and did not respond to IL-3 (as assessed by STAT5 phosphorylation), the overnight incubation with IL-4 (especially if associated with IL-3) upregulated CD123 expression. IL-3-activated JAK2-STAT5 pathway inhibitors reduced the CCL17 production in response to IL-3 and IL-4, but not to IL-4 alone. Interestingly, monocytes isolated from allergen-sensitized asthmatic patients exhibited a higher expression of CD123. Taken together, our data show that the JAK2-STAT5 pathway modulates both basophil and monocyte effector responses. The coordinated activation of STAT5 and STAT6 may have a major impact on monocyte alternative activation *in vitro* and *in vivo*.

Keywords

Allergy; Basophils; CCL17; Monocytes; JAK-STAT

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

Introduction

Type 2 immune responses are involved in conditions as diverse as allergy, tissue remodelling, parasitic infections [1, 2] and defence against animal venoms [3-5]. They are marked by the coordinated expression of Th2 cytokines (e.g. IL-4, IL-13, and IL-5) produced by several immune cell subsets (e.g. basophils, mast cells, innate lymphoid cells, NKT cells, and T lymphocytes). Interestingly, some *in vivo* models have uncovered a non-redundant role for basophils as a unique source of these cytokines [6].

Basophils are circulating granulocytes that account for less than 1% of blood leukocytes. Both human and mouse basophils express the high affinity IgE receptor (FcεRI). In response to IgE-dependent stimulation, they release a variety of preformed and *de novo* synthesized mediators, namely histamine, LTC₄ and the Th2 cytokines IL-4 and IL-13 that are hallmarks in allergic disease. In addition, circulating basophils express the IL-3 receptor α chain (IL-3Rα or CD123) for which binding of IL-3 is known to enhance every function of these cells, let alone its capacity to act on precursor cells to promote basopoiesis [7, 8]. Notwithstanding their rarity, basophils infiltrate inflamed tissue in several human diseases [9-12] and play a unique role in the development of some *in vivo* models of type 2 inflammation [6, 13, 14]. In a murine model of IgE-mediated chronic allergic inflammation (IgE-CAI) [15] as well as in the context of skin infestation by *Nippostrongylus brasiliensis* larvae [16], basophil-derived IL-4 induces the alternative (M2) activation of tissue-infiltrating inflammatory monocytes. Recently, it was shown that human basophils modulate LPS-induced proinflammatory activation of human monocytes [17]. It is currently unknown whether and how human basophils could modulate human monocyte/macrophage alternative activation.

Inflammatory monocytes (expressing Ly6C in mice and CD14 in humans) and monocyte-derived macrophages (MDM) are highly versatile effector cells owing to their ability to polarize in response to a wide spectrum of stimuli [18, 19]. Specifically, IL-4-induced STAT6 activation mediates the alternative activation of monocytes/macrophages which is characterized by increased expression of phagocytic receptors (e.g. the mannose receptor CD206) and the CCR4-binding chemokines CCL17/Thymus and activation regulated chemokine (TARC) and CCL22/Macrophage-derived chemokine (MDC) [18, 20]. These two chemokines have been linked to type 2 immune disorders such as bronchial asthma [21-24] and atopic dermatitis [25-28] owing to their ability to recruit CCR4-expressing Th2 lymphocytes. Thus, identifying the molecular and cellular mechanisms that regulate human monocyte/macrophage alternative activation may be relevant for understanding their basic biology as well as type 2 immune disorders.

Using a human basophil-monocyte co-culture model, we found that IL-3 and basophil-derived IL-4/IL-13 induced CCL17 production by human monocytes. We provide evidence that the IL-3-JAK2-STAT5 pathway is directly involved in monocyte alternative activation and synergizes with IL-4-activated STAT6 in inducing CCL17 expression and chromatin remodelling at the *CCL17* locus. The translational relevance of these findings was evaluated by showing that monocytes isolated from allergen-sensitized asthmatic patients express higher levels of CD123 compared to monocytes isolated from healthy controls.

Results

CCL17 production in human basophil-monocyte co-culture

To investigate the hypothesis that human basophils can modulate monocyte alternative activation, we purified the two cell types from the same donor and co-cultured them at basophil:monocyte ratios of 1:5, 1:20 and 1:50. Cells were stimulated with different combinations of IL-3 and anti-IgE, the latter used at two different concentrations known to preferentially induce the release of IL-4 or histamine (i.e. 10 and 100 ng/ml, respectively) [29]. Although anti-IgE alone was insufficient to stimulate detectable CCL17 production, its combination with IL-3 resulted in relatively high levels of CCL17 (Fig. 1A). Although not shown herein, we also tested for induced expression of CD206 as a marker of monocyte alternative activation. However, IL-3 alone (in the absence of basophils) upregulated CD206 on monocytes (data not shown).

Importantly, purified basophils stimulated with IL-3 and anti-IgE did not release CCL17 (Supporting Information Fig. 1A), thus indicating monocytes as the source of this chemokine. IL-3 and anti-IgE induced low to no production of CCL17 by purified monocytes, implying the requirements for basophils in the co-cultures (Supporting Information Fig. 1B). Interestingly, CCL17 production was highest at an anti-IgE concentration of 10 ng/ml, which is the optimal dose for inducing cytokines from basophils [29]. This effect was evident at every basophil to monocyte ratio tested, suggesting the involvement of IL-4/IL-13 in mediating this monocyte response (Fig. 1A and Supporting Information Fig. 2). To test this hypothesis, we duplicated these conditions but in the presence of neutralizing anti-IL-4, anti-IL-13, a combination of both or an isotype control. The concurrent blocking of IL-4 and IL-13 completely abolished the effect of anti-IgE and resulted in a marked reduction of CCL17 production (Fig. 1B).

IL-3 and IL-4 synergistically modulate monocyte CCL17 expression

The results obtained with the co-culture model suggested that IL-3 and anti-IgE acted on basophils to induce IL-4 and IL-13 release, which in turn promoted CCL17 production by monocytes. Nonetheless, we reasoned that IL-3 may also be directly modulating the monocyte response and therefore proceeded to investigate this hypothesis. We stimulated monocytes with different combination of recombinant cytokines (i.e. IL-3, IL-4, IL-13 and IFN- γ). IL-4 but not IFN- γ induced CCL17 production from monocytes. Surprisingly, although IL-3 alone induced low to no production of CCL17, the combination of IL-3 and IL-4 markedly increased CCL17 expression (Fig. 2A). The same pattern of response was observed with MDM (Supporting Information Fig. 3) or when IL-13 was used instead of IL-4, although IL-13 was less potent in inducing CCL17 and it did not increase CCL17 production when added to monocytes stimulated with IL-3 and IL-4 (data not shown). A time-course analysis of *CCL17* mRNA expression in monocytes stimulated with IL-3 and IL-4 showed a maximal expression at 12 hours and a decrease at 24 hours (Fig. 2B). We also tested the expression of CD206. Although IL-3 and IL-4 independently increased *CD206* mRNA expression, their combination markedly upregulated *CD206* (Supporting Information Fig. 4).

IL-3 belongs to a cytokine family that comprise also GM-CSF and IL-5 [30]. When these cytokines were used instead of IL-3, we observed that GM-CSF but not IL-5 increased CCL17 production when combined with IL-4 (Supporting Information Fig. 5). Furthermore, no production of IL-10 or IL-12p70 was observed in any of these cultures (Supporting Information Fig. 6), implying that the synergism between IL-3 (or GM-CSF) and IL-4 is specific for CCL17, a marker of monocyte/macrophage alternative activation.

Modulation of monocyte responsiveness to IL-3

The IL-3 receptor consists of a common β subunit (shared with GM-CSF and IL-5) and an IL-3 specific α subunit (CD123). The binding of IL-3 induces receptor heterodimerization and activation of the JAK2-STAT5 signaling pathway [30]. To identify the molecular basis of the synergism between IL-3 and IL-4, we performed short-term stimulations of freshly isolated monocytes with IL-3 and/or IL-4 and assessed STAT5 and STAT6 phosphorylation. Surprisingly, we did not identify relevant phosphorylation of STAT5 in any of the tested conditions (Supporting Information Fig. 7A). Furthermore, IL-3 did not modulate IL-4-induced STAT6 phosphorylation (Supporting Information Fig. 7B and C). Thus, we reasoned that long-term incubation with IL-4 could modulate monocyte responsiveness to IL-3. Accordingly, IL-3 stimulation of monocytes preincubated overnight (12 hours) with IL-4 induced STAT5 phosphorylation (Fig. 2C). On the contrary, overnight preincubation with IL-3 did not modify the response to IL-4 (Supporting Information Fig. 8).

Confirming previous reported data [31], freshly isolated monocytes weakly expressed CD123, which in turn was markedly upregulated by overnight treatment with IL-4 (particularly in combination with IL-3) (Fig. 2D and E). These results were paralleled by STAT5 phosphorylation induced by IL-3 stimulation in monocytes preincubated overnight with IL-4, IL-3, or a combination of both (Supporting Information Fig. 9). No upregulation of CD123 was observed when IFN- γ , IL-10 or M-CSF were used instead of IL-4 and IL-3 (Supporting Information Fig. 10).

The JAK2-STAT5 signaling pathway is required for CCL17 production

To assess the specific contribution of the JAK2-STAT5 signaling pathway to CCL17 production, we stimulated monocytes with IL-3 and IL-4 in the presence of different concentrations of pimozone (a STAT5 inhibitor) or TG101348 (a JAK2 inhibitor). To ascertain the specificity of these inhibitors, we stimulated monocytes also with IL-4 alone to identify those concentrations that do not affect CCL17 production in response to IL-4. Concurrently, we assessed the phosphorylation of STAT5 and STAT6 respectively in response to IL-3 and IL-4 in the presence of these inhibitors. Pimozone reduced CCL17 production in response to IL-3 and IL-4 (Fig. 3A). Although pimozone was reported to reduce STAT5 constitutive phosphorylation of chronic myelogenous leukemia cells [32], in our model it did not modulate IL-3- or IL-4-induced STAT5 and STAT6 phosphorylation, respectively (Supporting Information Fig. 11A-D). TG101348 also reduced CCL17 production in a dose-dependent manner. However, at a concentration of 250 nM it reduced the response to IL-4 (Fig. 3B). Consistently, at this concentration it reduced the phosphorylation of both STAT5 and STAT6, while at lower concentrations it preferentially modulated STAT5 (Supporting Information Fig. 11E-H). Interestingly, the specificity of

TG101348 is concentration-dependent, with IC₅₀s for JAK1, JAK2 and JAK3 being respectively 105, 3 and 1002 nM. As IL-4-induced STAT6 phosphorylation is mediated by JAK1 in human monocytes [33], it is conceivable that TG101348 at 250 nM inhibits both JAK2 and JAK1, while at lower concentrations is more specific for JAK2.

IL-3 and IL-4 modulate histone acetylation and transcription factor binding at the *CCL17* locus

IL-4-induced *CCL17* transcription is associated with STAT6 binding at three genomic regions in the first intron: two of them located ≈200 bp [34] and another one located ≈2500 bp [35] upstream of the translational start site (respectively R1 and R2, Fig. 4A). In addition, we identified 2 putative STAT5 binding sites within 2000 bp of the *CCL17* transcriptional start site (TSS) (R4 and R5, Fig. 4A) by MatInspector software analysis, and another putative STAT binding site located ≈1800 bp downstream of *CCL17* TSS by visual inspection of the first intron (R3, Fig. 4A). Thus, we asked whether IL-3 and IL-4 could modulate STAT5 and STAT6 binding and histone H3 acetylation (H3ac), which is a marker of actively transcribed genes [36], in these regions. Furthermore, we assessed H3ac in an additional region (Nuc1, Fig. 4A) located between 1800 and 1918 bp upstream of the *CCL17* TSS. Consistent with gene expression data, IL-3 and IL-4 treatment increased H3ac both downstream (R2 and R3) and upstream (Nuc1) of the *CCL17* TSS (Fig. 4B). IL-4 stimulation correlated with the recruitment of STAT6 at R1, R2 and also R3. Surprisingly, the combination of IL-3 and IL-4 reduced STAT6 binding at these regions (Fig. 4C). Furthermore, no association of STAT5 with any of the tested regions (R1-R5) was observed (data not shown).

Monocytes isolated from asthmatic patients express higher levels of CD123

Our results suggest that the IL-3-JAK2-STAT5 pathway modulates monocyte alternative activation *in vitro*. To verify the *in vivo* relevance of this finding, we recruited allergen-sensitized asthmatic patients and age- and sex-matched healthy controls (Supporting Information Table 1). Monocytes were isolated from both groups and their expression of CD123, CD206 and *CCL17* was assessed. *CCL17* mRNA was increased only in one patient (interestingly the one with the highest eosinophil blood count and serum IgE levels), while it was undetectable in others (data not shown). Critically, monocytes isolated from asthmatic patients expressed higher levels of CD123 as assessed by flow cytometry and real time RT-PCR (Fig. 5, respectively A and B). No significant differences in *CD206* mRNA expression were observed (Fig. 5C).

Discussion

In this study we demonstrate that human basophils activated with IL-3 and anti-IgE induce *CCL17* production by human monocytes. This effect is mediated by basophil-derived IL-4 and IL-13. In addition, IL-3 (as well as GM-CSF) acts on monocytes and synergizes with IL-4 to increase *CCL17* production. Freshly isolated monocytes do not express CD123 or respond to IL-3 (as assessed by STAT5 phosphorylation). However, overnight incubation with IL-4 (especially if associated with IL-3) upregulates CD123 and makes monocytes responsive to IL-3. Although the IL-3-activated JAK2-STAT5 pathway is required for

modulating CCL17 production, IL-3 reduces IL-4-induced STAT6 binding at the *CCL17* locus without inducing STAT5 binding. The clinical relevance of these findings is supported by the observation that monocytes isolated from allergen-sensitized asthmatic patients express higher levels of CD123 compared to healthy donors.

In our co-culture model basophil-derived IL-4 and IL-13 were both required for maximal production of CCL17, while the addition of IL-13 to purified monocytes stimulated with IL-3 and IL-4 did not greatly improved CCL17 production. This apparent discrepancy may be explained by considering that IL-4 receptor α associates with common cytokine receptor γ chain to form the type I receptor complex and also couples with IL-13 receptor $\alpha 1$ to form the type II receptor complex. While IL-13 binds only the latter, IL-4 binds both [37]. Thus, it is conceivable that in the co-cultures IL-4 reached submaximal concentrations and IL-13 could bind its receptor complex. Instead, when purified monocytes were stimulated with supramaximal concentrations of IL-4, both receptor complexes were occupied and no additional effect of IL-13 could be observed.

IL-3 and its related family members GM-CSF and IL-5 modulate not only the production but also the effector functions of several hematopoietic cells [30]. For example, IL-3 acts on precursor cells to stimulate basopoiesis, but also on mature basophils to modulate mediator release and cytokine secretion [7, 8]. Murine bone marrow precursors treated with IL-3 differentiate into macrophages and basophils. The latter, especially in the absence SHIP, secrete high levels of IL-4, which in turn induces the alternative activation of macrophages [38]. Recently, it was shown that IL-3 upregulates CD206 expression on MDM [39]. Although previous studies reported that IL-3 modulates some functions of monocytes [40, 41], IL-3 and GM-CSF have been used mainly in combination with IL-4 to differentiate human monocytes into dendritic cells (DCs) after 5-7 days in culture [42, 43]. As the role of monocytes as effector cells has been recently reevaluated [44], our analysis focused on the short-term (less than 24 hours) effects of IL-3 and IL-4 on monocytes. We aimed also to characterize the role of the IL-3-JAK2-STAT5 pathway in the context of monocyte/macrophage polarization. Specifically, we demonstrate that the concurrent activation of STAT5 (mediated by IL-3 or GM-CSF) and STAT6 (mediated by IL-4) potentiates the production of CCL17, a chemokine relevant to allergic inflammation [21-28]. Although we focused on a small set of cytokines and surface receptors, it will be interesting to perform a genome-wide analysis to evaluate in depth the contribution of STAT5 and STAT6 to monocyte/macrophage activation.

Both IL-3 and GM-CSF induce human monocyte differentiation into DCs in association with IL-4 [42, 43]. However, some differences exist between these two cytokines. IL-3-differentiated DCs have a greater ability than GM-CSF-differentiated DCs to polarize CD4 T lymphocytes toward a Th2 phenotype [42]. It is tempting to speculate that IL-3 and IL-4 mediate monocyte/macrophage alternative activation in the short-term and monocyte differentiation toward Th2-inducing DCs in the long-term. Thus, it will be interesting to assess whether basophils can modulate monocyte differentiation into DCs in a long-term co-culture model. Although several lines of evidence suggest that basophils are not major APCs *in vitro* and *in vivo* [6, 13], basophils activated by IL-3 and anti-IgE may provide essential

mediators (e.g. IL-4 and IL-13) for differentiating monocytes into Th2-inducing DCs. These experiments will give a comprehensive picture of human basophil-monocyte interaction.

In this study we focused on cytokines that signal through the common β chain and activate the JAK2-STAT5 pathway. Another cytokine that activates STAT5 is thymic stromal lymphopoietin (TSLP) [45]. TSLP is produced by epithelial cells following stimulation with microbial and inflammatory stimuli and acts on cells expressing the TSLP receptor complex (a heterodimer composed of TSLP receptor [TSLPR] and IL-7 receptor α [IL-7R α]) [37]. Several immune cell subsets are responsive to TSLP. In particular, TSLP-activated DCs induce Th2 differentiation and type 2 immunity *in vitro* and *in vivo* [46, 47]. Interestingly, TSLP and IL-4 or IL-13 synergize to induce CCL17 production by mouse macrophages [48]. These results strengthen our model according to which the combined action of STAT5- and STAT6-activating cytokines modulates monocyte/macrophage alternative activation. Nonetheless, in our experience freshly isolated human monocytes do not express TSLPR nor do they phosphorylate STAT5 in response to TSLP (unpublished observations). Further studies are required to define human monocyte responsiveness to TSLP.

Monocyte/macrophage activation is ultimately linked to the binding of transcription factors to responsive elements and the subsequent chromatin remodelling that modulates gene expression [49]. Although STAT6 binds at *CCL17* locus and induces its expression [34, 35], IL-3 and IL-4 increased H3ac but unexpectedly reduced STAT6 binding. In addition, we could not identify STAT5 binding at the *CCL17* locus. A hypothesis that may explain these discrepancies takes into consideration the nuclear localization of JAK2. Indeed, hematopoietic cells express JAK2 also in the nucleus where it mediates the phosphorylation of tyrosine 41 of histone H3 (H3Y41ph). This event, in turn, mediates the displacement of the heterochromatin protein HP1 α and induces gene expression [50]. Interestingly, H3Y41ph has been mapped at a subset of active promoters in the absence of STAT5 binding [51]. It is tempting to speculate that the addition of IL-3 to IL-4 switches *CCL17* regulation from STAT6-dependent to JAK2-dependent. However, we could not detect JAK2 in nuclear extracts of freshly isolated monocytes or monocytes stimulated overnight with IL-4 and/or IL-3 (Supporting Information Fig. 12). Further studies are required to define the global profiles of STAT5 and STAT6 binding sites and how they relate to chromatin regulatory elements in human monocytes.

Akin to many studies focused on human immune cells, ours do not provide mechanistic evidence *in vivo* that the IL-3-JAK2-STAT5 pathway is involved in monocyte alternative activation, nor that basophils interacts with monocytes. Nevertheless, we show that monocytes isolated from allergen-sensitized asthmatic patients express higher levels of CD123 but not CD206, a marker of alternative activation. Thus, it is unlikely that increased expression of CD123 reflects *in vivo* exposure of monocytes to IL-4 and/or IL-3. Rather, it may suggest increased mobilization of CD14⁺ bone marrow monocytes, which have been shown to express CD123 [52]. Indeed, increased mobilization of bone marrow precursors has been observed in several inflammatory conditions [53-57]. Further studies are required to investigate this hypothesis.

Taken together, our results confirm and extend those obtained in mouse models of type 2 inflammation according to which basophils induce monocyte alternative activation. We provide evidence that the IL-3-JAK2-STAT5 pathway is involved not only in basophil activation, but also in monocyte alternative activation. We also demonstrate that monocyte expression of CD123 is upregulated in allergen-sensitized asthmatic patients. Although the reasons for increased CD123 expression are unclear at present, it suggests that a “monocyte gene signature” might exist in atopic asthma and perhaps other allergic diseases. The definition of a “monocyte gene signature” will require a translational approach in which data arising from *in vitro* studies are tested in appropriate cohorts of patients, an approach that already led to the characterization of the molecular phenotypes of asthma [58]. Thus, studying how monocyte alternative activation is shaped by STAT5 and STAT6 signaling pathways may give new insights into both monocyte biology and the molecular signatures of allergic diseases.

Materials and methods

Subjects

The study protocol involving the use of human blood cells was approved by the Ethics Committee of the University of Naples Federico II and by the Western Institutional Review Board (Seattle, Wash). Written informed consent was obtained from blood donors.

Cells were isolated from buffy coats of healthy donors. In selected experiments, monocytes were isolated from peripheral blood of asthmatic patients and age- and sex-matched healthy controls. Six allergen-sensitized asthmatic patients were enrolled in this study (Supporting Information Table 1), 5 of them were treatment-naïve and 1 was taking inhaled steroid and β_2 agonists at the time of blood draw. Asthma severity was assessed by clinical examination and spirometry (FEV_1 and FEV_1/FVC ratio [% of predicted values]). Atopy was assessed by skin prick testing to common aeroallergens, eosinophil blood count and serum IgE levels. Both patients and healthy controls were nonsmokers and had no respiratory tract infections in the 6 weeks before the study.

Cell isolation and culture

Basophils and monocytes were enriched as previously described [59]. Alternatively, blood was layered onto Histopaque-1077 (Sigma-Aldrich) and mononuclear cells were collected at the interface. Basophils and monocytes were further purified with Basophil Isolation Kit II and CD14 Microbeads, respectively (Miltenyi Biotec). Purity of cell preparations was > 98% as assessed by alcian blue staining (basophils) or flow cytometry (monocytes).

Cells were cultured in cIMDM-5 (IMDM, 5% FCS, 1x non essential amino acids, 1x UltraGlutamine, 25 mM HEPES, 5 μ /ml gentamicin [Lonza]) in 96-well flat-bottom plates in a final volume of 250 μ l. Monocytes (2×10^5) were added to each well and cultured alone and with basophils added at the indicated basophil to monocyte ratio. For experiments involving flow cytometry, cells were cultured in 1.5 ml tubes, then spun down and collected for the subsequent staining protocols. For monocyte-derived macrophages (MDM), monocytes were cultured (5×10^4 cells/well in 96 well flat bottom plates) for 7 days in

cRPMI-10 (RPMI, 10% FCS, 1x UltraGlutamine, 5 µ/ml gentamicin [Lonza]) + M-CSF 50 ng/ml (Miltenyi). Cells were treated with different combinations of: IL-3, IFN-γ, IL-10 (PeproTech), IL-4, IL-13, GM-CSF, IL-5 (Miltenyi Biotec), anti-human IL-4, anti-human IL-13, rat IgG1 κ isotype control (Biolegend), pimozide (Merck Millipore), and TG101348 (Selleck Chemicals). Polyclonal anti-IgE was kindly provided by Robert G. Hamilton (Johns Hopkins Asthma & Allergy Center, Baltimore, USA).

ELISA

Cytokine concentrations were measured in cell-free supernatants using commercially available ELISA kits for CCL17 (R&D Systems), and IL-10 and IL-12p70 (eBioscience). Standard curves were generated with a Four Parametric Logistic curve fit and data were analysed using MyAssays Analysis Software Solutions (www.myassays.com).

Flow cytometry

Flow cytometry experiments were performed with purified monocytes. For surface staining, cells were stained (20 minutes at 4°C) in PBS + 10% human AB serum (Lonza) + 0.05% NaN₃ (Staining buffer, SB). For phosphoprotein staining, cells were rested in cIMDM-5 for 1 hour and stimulated with the indicated cytokines for 15 minutes. Then, cells were fixed with paraformaldehyde (EM-grade, Electron Microscopy Sciences) (final concentration 1.5%) and permeabilized with absolute ice-cold methanol as previously described [60]. Cells were stained (30 minutes at room temperature) in SB. The following antibodies were used: anti-CD123 FITC (clone AC145, dilution 1:20), anti-CD206 FITC (clone DCN228, dilution 1:20) (Miltenyi Biotec), anti-phospho-STAT5 AlexaFluor 647 (clone 47, dilution 1:20) and anti-phospho-STAT6 AlexaFluor 647 (clone 18, dilution 1:20) (BD Biosciences). Samples were acquired on a BD LSRFortessa and analyzed using FACSDiva Software. Data are expressed as percentage of positive cells and median fluorescence intensity (MFI) of positive cells. Gating strategies for surface staining and phosphoprotein staining are shown in Supporting Information Fig. 13 and Fig. 14, respectively.

RNA isolation and real time RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Reverse transcription of 1 µg of total RNA was performed using SuperScript III (Invitrogen), following the manufacturer's instructions. Real time RT-PCR was performed in triplicate by using iQ SYBR Green Supermix on iCycler real time detection system (Bio-Rad). Relative quantification of gene expression was calculated by the C_t (relative expression $\times 10^4$) method. Each C_t value was normalized to the respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) C_t value. The following primer pairs were used: *CCL17* forward 5'-tccaggatgccatcgctttt-3'; *CCL17* reverse 5'-tcctcactgtggctcttct-3'; *CD123* forward 5'-ggcgaggagaatctgacctg-3'; *CD123* reverse 5'-gtactgttgacgcctgttg-3'; *CD206* forward 5'-cgatccgacccttccttgac-3'; *CD206* reverse 5'-tgtctccgttcattgac-3'; *GAPDH* forward 5'-cgctctgctcctctgttc-3'; *GAPDH* reverse 5'-ttgactccgaccttcaccttc-3'.

Chromatin immunoprecipitation assay (ChIP)

Upon protein-DNA cross-linking, cells were lysed and sonicated to achieve chromatin fragments ranging between 200 and 1000 bp in size. The lysates were incubated with anti-H3Ac (Millipore), anti-STAT5 (Cell Signaling Technologies), anti-STAT6 (Santa Cruz Biotechnology) or isotype controls, and then complexes were isolated using Dynabeads Protein A (Invitrogen). Immunoprecipitates were extensively washed and then eluted with freshly prepared 1% SDS, 0.1 M NaHCO₃ buffer. After reversion of cross-linking and deproteinization, DNA was purified with QIAquick PCR purification kit (Qiagen) followed by real time PCR analysis. The following primer pairs were used: R1 forward 5'-cagctgtgcgtggaggctttca-3'; R1 reverse 5'-tcctccctagaccagtgaagtcgaaga-3'; R2 forward 5'-tgcaccagccttgaactgaaccag-3'; R2 reverse 5'-gctacacaactgcaaggacagctgatta-3'; R3 forward 5'-ccacccagcctatagttag-3'; R3 reverse 5'-tagtctccagctggctcagg-3'; R4 forward 5'-cgtctgaacacgggagat-3'; R4 reverse 5'-tggtgagctcatgtctctg-3'; R5 forward 5'-gcctaccatgtgccgagata-3'; R5 reverse 5'-agattacatcttcagtgaagtcaa-3'; Nuc1 forward 5'-gcctaccatgtgccgagata-3'; Nuc1 reverse 5'-tgtttactatctgtctccactg-3'.

Western blot analysis

For pSTAT5 detection, cells were lysed according to the manufacturer's protocol. Cytoplasmic and nuclear extracts were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). 10 µg of protein lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with the following antibodies: anti-phospho-STAT5 (#9351, dilution 1:1000), anti-STAT5 (#4459, dilution 1:1000), anti-JAK2 (#3230, dilution 1:1000) (Cell Signaling Technology), anti-α-tubulin (#T9026, dilution 1:5000) (Sigma) and anti-lamin A/C (#sc-6215, dilution 1:500).

Statistical analysis

Statistical analysis was performed with Prism 6 (GraphPad Software). *p* values were calculated with paired *t* test or repeated measure one-way ANOVA corrected for multiple comparisons unless otherwise indicated. Flow cytometry percentage data were analysed by using Wilcoxon matched-pairs signed rank test or Friedman test corrected for multiple comparisons. *p* < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMI	body mass index
DCs	dendritic cells
FEV₁	forced expiratory volume in the 1 st second
FVC	forced vital capacity
H3ac	acetyl-histone H3
JAK	just another kinase
MDM	monocyte-derived macrophages
pSTAT	phospho-STAT
TSS	transcriptional start site

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Figure 1.

CCL17 production in basophil-monocyte co-cultures. (A, B) Basophils and monocytes were co-cultured at a 1:5 ratio and stimulated with αIgE (10 or 100 ng/ml) and IL-3 (5 ng/ml) for 24 hours. CCL17 levels were measured by ELISA in cell-free supernatants. (B) Co-cultures were pretreated with IgG isotype control (Iso), αIL-4, αIL-13 or αIL-4 + αIL-13 15 minutes before adding stimuli. Data are shown as the median, the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 6 independent experiments. ** $p < 0.01$ determined by repeated measure one-way ANOVA with Tukey's post hoc test.

Figure 2.

Monocyte responsiveness to IL-3. (A) Monocytes were stimulated with IL-4 (10 ng/ml), IL-3 (0.2, 1 or 5 ng/ml) and IFN- γ (100 U/ml) for 24 hours. CCL17 levels were measured by ELISA in cell-free supernatants. (B) Monocytes were stimulated with IL-4 (10 ng/ml), IL-3 (1 ng/ml) and a combination of both. *CCL17* mRNA levels are expressed as relative expression (normalized to GAPDH) $\times 10^4$. Data are representative of two independent experiments. (C) Monocytes were cultured overnight with (ON + IL-4) or without (ON) IL-4 (10 ng/ml). Then, monocytes were stimulated with IL-3 (1 ng/ml). STAT5 phosphorylation was assessed by flow cytometry. Results are expressed as percentage of pSTAT5 positive cells. (D, E) Monocytes were cultured overnight without (ON) or with IL-4 (10 ng/ml), IL-3 (1 ng/ml), or both. CD123 expression was assessed by flow cytometry on cultured as well as freshly isolated (0h) monocytes. Results are expressed as (D) percentage and (E) MFI of CD123 positive cells. (A, C-E) Data are shown as mean + SEM of 4 (A), 6 (C) or 9 (D, E) independent experiments. * $p < 0.05$, ** $p < 0.01$ determined by repeated measure one-way ANOVA with Tukey's post hoc test (A, E), Wilcoxon matched-pairs signed rank test (C) and Friedman test with Dunn's post hoc test (D).

Figure 3.

Effect of the inhibition of the JAK2-STAT5 pathway on CCL17 production. Monocytes were preincubated with different concentrations of (A) pimozone (500 or 50 nM) or (B) TG101348 (250, 125 or 62.5 nM) and then stimulated with IL-4 (10 ng/ml) and IL-3 (1 ng/ml) for 24 hours. CCL17 levels were measured by ELISA in cell-free supernatants. (A, B) Data are shown as mean + SEM of 5 independent experiments. ** $p < 0.01$ determined by repeated measure one-way ANOVA with Sidak's post hoc test.

Figure 4.

H3ac and STAT6 binding at the *CCL17* locus. (A) Schematic representation of the *CCL17* locus, including the transcriptional start site (TSS, chr16:57438679) and the translational start site (ATG, chr16:57447845). The positions of the tested regions (R1-R5, Nuc1) are indicated as relative to TSS. (B, C) Monocytes were cultured overnight without (CTRL) or with IL-4 (10 ng/ml), IL-3 (1 ng/ml) and a combination of both. H3ac and STAT6 binding was assessed by ChIP at Nuc1, R2, R3 (B) and R1, R2, R3 (C), respectively, and expressed as percent of input (B, C). Data are shown as mean + SEM of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ determined by repeated measure one-way ANOVA with Sidak's post hoc test.

Figure 5.

CD123 and CD206 expression in monocytes isolated from asthmatic patients. CD123 and CD206 expression was assessed in monocytes isolated from 6 allergen-sensitized asthmatic patients (Patients) and 6 healthy controls (Controls) by means of flow cytometry (A) and real time RT-PCR (B, C). Results are expressed as (A) percentage of CD123 positive cells and (B, C) relative expression of *CD123* and *CD206* mRNA (normalized to GAPDH) $\times 10^4$. (A-C) Data are shown as the median, the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 6 Patients and 6 Controls. * $p < 0.05$ determined by Wilcoxon matched-pairs signed rank test.