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CUTTING EDGE

Cutting Edge: IL-5 Primes Th2 Cytokine-Producing Capacity in Eosinophils through a STAT5-Dependent Mechanism[1]

Yuechun Zhu, Luqiu Chen,* Zan Huang,* Serhan Alkan,† Kevin D. Bunting,‡§ Renren Wen,¶ Demin Wang,¶‖ and Hua Huang[2]**

Both type-2 CD4$^+$ Th cells (CD4$^+$ Th2) and type-2 innate effector cells play critical roles in generating type-2 immunity that can either be protective against parasitic infection or cause tissue damage in allergy and asthma. How innate effector cells acquire the capacity to produce Th2 cytokines is not entirely known. We previously showed that IL-4 induced differentiation of Th2 cytokine-producing eosinophils. To determine whether other Th2 cytokines can also induce Th2 cytokine-producing capacity in innate effector cells, we cultured bone marrow progenitor cells in the presence of various Th2 cytokines. IL-5, but not IL-13 or IL-25, primed bone marrow progenitor cells to differentiate into robust IL-4-producing cells. The majority of IL-4-producing cells induced by IL-5 were eosinophils. Importantly, IL-5 completely depended on STAT5 to promote IL-4-producing capacity in eosinophils. Thus, our study demonstrates that IL-5 functions as a potent factor that drives bone marrow progenitor cells into IL-4-producing eosinophils. The Journal of Immunology,* 2004, 173: 2918–2922.

Innate cells have been considered important effector cells in mediating allergic inflammation and in protecting against helminth infection (1, 2). Eosinophils can cause tissue damage by releasing inflammatory mediators from their granules, such as major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin (3). In addition, eosinophils are also an excellent source of inflammatory cytokines (4). The importance of IL-4-producing eosinophils in generating protective immunity against parasitic infection has been recently established (5). In the parasitic model, the generation, expansion, and maintenance of IL-4-producing eosinophils are essential for expelling helminth infection (5). We recently have reported that eosinophils produced the majority of Th2 cytokines in the late-phase response of allergic airway inflammation (6). We have further shown that IL-4 is able to drive differentiation and expansion of IL-4-producing eosinophils (6). However, it remains unknown whether other types of Th2 cytokines can direct bone marrow progenitor cells to differentiate into IL-4-producing innate cells.

IL-5 is a critical factor for eosinophils differentiation, activation, survival, and recruitment to the sites of inflammation (7). IL-5 exerts its functions through stimulating IL-5R α-chain and β-chain, also shared by IL-3 and GM-CSF. Stimulation of the IL-5R activates multiple signaling pathways, including the Jak-STAT pathway. IL-5 induces phosphorylation of Jak2, STAT1, and STAT5 (8, 9), which includes STAT5a and STAT5b — the two share 95% homology in amino acid sequences (10). Studies of mice deficient in both STAT5a and STAT5b have shown that the absence of both molecules reduces the number of IL-5-induced bone marrow colonies (11). However, it is not known whether IL-5 possesses the capacity to drive bone marrow progenitor cells into IL-4-producing cells. In addition, the biological role of STAT5a and STAT5b in IL-5 signaling remains unclear.

In this study, we examined whether other types of Th2 cytokines, in addition to IL-4, can induce differentiation of IL-4-producing innate effector cells from bone marrow progenitor cells. We found that IL-5, but not IL-13 or IL-25, was able to induce development of IL-4-producing eosinophils independent of IL-4. Intriguingly, the ability of IL-5 to drive bone marrow progenitor cells primarily into IL-4-producing eosinophils completely depended on STAT5a/b. Thus, our study demonstrates a novel STAT5-dependent role for IL-5 in driving differentiation of Th2-type eosinophils.

Materials and Methods

Mice

C57BL/6 and STAT6$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). STAT5a/b$^{-/-}$ mice on C57BL/6 background (backcrossed to C57BL/6 background for greater than six generations) as described (12). The IL-4/GFP reporter mice (13) are maintained as knockin GFP homozygous

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[3] Abbreviations used in this paper: 4GFP hom, homozygous for a knockin GFP; 4GFP het, heterozygous for a knockin GFP; SCF, stem cell factor; WT, wild type.

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(4GFP hom)[3] and as knockin GFP heterozygous (4GFP het) mice according to guidelines approved by the Loyola Animal User Committee.

Th2 priming

For priming of $CD4^+$ Th2 cells, lymph node cells were depleted of $CD8^+$ cells, $B220^+$ cells, and IA^{b+} cells by negative selection using magnetic beads. Primary stimulation of $CD4^+$ T cells was conducted as described previously (14). Briefly, naive $CD4^+$ T cells (10^6) in the presence of 10^7 irradiated T-depleted spleen cells from C57BL/6 mice with anti-CD3 (2C11, 3 μg/ml), anti-CD28 (3 μg/ml), anti-IL-12 Ab (C17.8, 10 μg/ml), IL-2 (10 U/ml), and IL-4 (5 ng/ml, BD Pharmingen, San Diego, CA) or IL-5 (20 ng/ml) for 5 days.

Methylcellulose culture and ELISA

Bone marrow lineage marker-negative and GFP-negative (lin^-GFP^-) cells were prepared as described previously (6) and seeded (2×10^4/dish) in 1% methylcellulose medium (Methocult M3234; StemCell Technologies, Vancouver, Canada). Stem cell factor (SCF) (25 ng/ml) alone or plus IL-4 (5 ng/ml), IL-5 (20 ng/ml), IL-13 (10 ng/ml), or IL-25 (20 ng/ml) (PeproTech, Rocky Hill, NJ) were added to the cultures. IL-4, IL-5, or IL-13 proteins were measured by using commercial ELISA detection kits (BD Pharmingen).

FACS sorting

IL-4- and IL-5-treated cells were harvested at day 9 and stimulated with PMA (10 ng/ml) and ionomycin (1 mM) overnight. GFP^+ cells were electronically sorted by using a BD FACStar^{Plus} instrument (BD Pharmingen). For morphological analysis, GFP^+ cells were collected in a test tube and spun onto a glass slide for May Grunwald Giemsa or toluidine blue staining.

Statistical analysis

All the error bars in this report represent SD. The differences between two samples were analyzed with Student's *t* test.

Results and Discussion

IL-5, but not IL-13 and IL-25, induces differentiation of IL-4-producing eosinophils

We previously showed that IL-4 induced differentiation and expansion of Th2 cytokine-producing eosinophils (6). To test whether other Th2 cytokines can induce Th2 cytokine-producing capacity in innate cells, we prepared bone marrow progenitor cells that had not yet possessed the capacity to produce IL-4 by taking advantage of the use of the IL-4/GFP heterozygous reporter (4GFP het) mice. Bone marrow cells from 4GFP het mice were depleted of mature cells and used as progenitor cells (lin^-). We further isolated progenitor cells that were not producing IL-4 by FACS sorting on the GFP^- progenitor cells (lin^-GFP^-). lin^-GFP^- bone marrow progenitor cells were cultured in 1% methylcellulose in the presence of SCF plus IL-4, IL-5, IL-13, or IL-25 for 9 days. Consistent with previous observation, IL-4 displayed potent effects in directing bone marrow progenitor cells to differentiate into IL-4-producing cells (Fig. 1*A*). Surprisingly, we found that IL-5 also possessed the ability to direct bone marrow progenitor cells into robust IL-4-producing cells (Fig. 1*A*). Compared with IL-4, IL-5 was a more potent inducer of IL-4-producing capacity in bone marrow-derived cells. The amounts of IL-4 protein produced by IL-5-primed cells were in the same magnitude to that by $CD4^+$ Th2 cells primed in vitro (Fig. 1*A*). These results suggest that IL-5 is a potent inducer of IL-4-producing capacity in bone marrow-derived cells.

$CD4^+$ Th2 cells are known to produce large amounts of IL-5 and IL-13. To analyze whether bone marrow-derived IL-4-producing cells can also produce IL-5 and IL-13, we measured these cytokine productions. Interestingly, both IL-4 and IL-5-primed cells produced less amounts of IL-13 than did $CD4^+$ Th2 cells (2- to 3-fold less, Fig. 1*C*). They produced even lower amounts of IL-5 compared with $CD4^+$ Th2 cells (Fig. 1*B*).

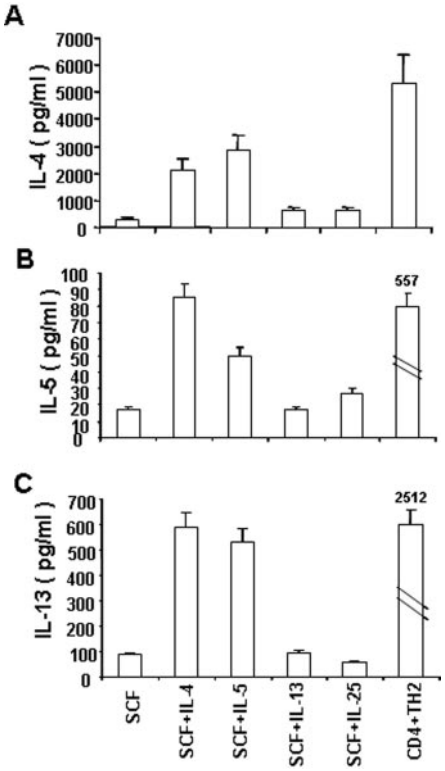

FIGURE 1. IL-4 and IL-5 induce differentiation of Th2 cytokine-producing cells. lin^-GFP^- cells from bone marrow of 4GFP het mice were cultured in 1% methylcellulose containing SCF alone or SCF plus IL-4 (5 ng/ml), IL-5 (20 ng/ml), IL-13 (10 ng/ml), or IL-25 (20 ng/ml) for 9 days. $CD4^+$ Th2 cells were used as positive controls. The resultant cells were washed extensively and stimulated (at 1×10^6 in 1 ml) with PMA (10 ng/ml) and ionomycin (1 μM) overnight. IL-4, IL-5, and IL-13 proteins were measured by ELISA. These results represent three independent experiments.

These results suggest that IL-4 and IL-5 may regulate Th2 cytokine gene transcription differently in innate cells than do they in $CD4^+$ Th2 cells.

To determine the identities of the IL-4-producing cells driven by IL-4 or IL-5, we primed lin^-GFP^- cells prepared from 4GFP het mice in the presence of SCF plus IL-4 or IL-5 for 9 days. The resultant cells were stimulated. Approximately, 5–10% of harvested cells expressed GFP and 3–6% of eosinophils expressed GFP (data not shown). GFP^+ cells, reporting for IL-4-producing cells, were isolated by FACS sorting. We achieved over 99% purity. Sixty-four percent of GFP^+ cells derived from IL-5-treated cultures were eosinophils compared with 61% of GFP^+ cells derived from IL-4-treated cultures (Table I). GFP^+ cells were negative for CD4 molecule and expressed mRNA for eosinophil-specific genes, such as CCR3, major basic protein, and eosinophil peroxidase (results not shown). We detected that around 6% of GFP^+ cells were mast cells in IL-5-treated culture, but did not identify mast cells in GFP^+ cells derived from IL-4-treated cultures. We also found that 5–16% of GFP^+ cells fitted into categories of neutrophils and monocytes in both IL-4- and IL-5-treated cultures (Table I). Unsorted cells used for morphologic analysis were either not stimulated or stimulated (Table I). We did not observe any difference in cell compositions between unstimulated and stimulated cells (results not shown). Although we detected around 5–15% of basophils in GFP^- cell populations that were treated

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Table I. *Morphology of GFP$^+$ cells induced by IL-4 and IL-5*[a]

		Eos	Mas	Neu	Mon	Bas
SCF	Unsorted	17.0 ± 0.4	35.3 ± 2.1	17.7 ± 45	28.5 ± 3.4	1.5 ± 0.4
	GFP$^+$	UD[b]	UD	UD	UD	UD
SCF + IL-4	Unsorted	32.8 ± 9.0	UD	9.4 ± 4.6	43.2 ± 8.1	14.6 ± 3.8
	GFP$^+$	61.8 ± 9.8	UD	12.6 ± 1.7	16.1 ± 3.6	UD
SCF + IL-5	Unsorted	31.2 ± 9.2	6.0 ± 1.5	36.4 ± 9.5	20.9 ± 9.8	5.5 ± 1.3
	GFP$^+$	64.2 ± 5.6	6.4 ± 0.5	15.5 ± 1.9	5.6 ± 2.8	UD

[a] lin-bone marrow cells (2×10^4) *of 4GFP het mice were seeded in the presence of SCF (25 ng/ml) or plus IL-4 (5 ng/ml) or IL-5 (20 ng/ml) for 9 days. Unsorted cells or GFP$^+$ cells sorted electronically from stimulated cultures were analyzed morphologically. Eos stands for eosinophils; Mas for mast cells; Neu for neutrophils; Mon for monocytes; and Bas for basophils.*
[b] UD, Undetectable

with either IL-4 or IL-5, we did not detect any basophils in GFP$^+$ cells (Table I). These results suggest that IL-5 drives bone marrow progenitor cells primarily into IL-4-producing eosinophils.

IL-4 induces differentiation of Th2-type innate cells independent of STAT6 signaling

STAT6 is essential in IL-4-induced differentiation of CD4$^+$ Th2 cells (15). To determine the role of STAT6 in IL-4-induced differentiation of IL-4-producing eosinophils, we cultured lin$^-$ bone marrow progenitor cells of wild-type (WT) and STAT6 deficient mice. STAT6$^{-/-}$ bone marrow progenitor cells differentiated into IL-4- and IL-13-producing as efficient as WT progenitor cells (Fig. 2). These data indicate that, in contrast to its role in differentiation of CD4$^+$ Th2 cells, STAT6 is not required for IL-4-mediated differentiation of Th2-type eosinophils.

IL-5 depends on STAT5a/b to induce IL-4-producing capacity in bone marrow-derived cells

IL-5 is not a known factor in driving development of IL-4-producing capacity in CD4$^+$ T cells. It is possible that IL-5 induces development of IL-4-producing bone marrow-derived cells via an IL-4-dependent mechanism. To rule out this possibility, we primed bone marrow progenitor cells of 4GFP hom mice (lack functional IL-4, and Ref. 6) with IL-5. We measured IL-13 protein in the resultant cells and found that the IL-4 deficient cells produced IL-13 proteins although the amounts were 50% of control cells (Fig. 3). IL-5 also drove bone marrow progenitor cells to differentiate into eosinophils independent of IL-4 (data not shown). These results indicate that IL-5 acts independent of IL-4, although the presence of IL-4 may enhance IL-5-driven development of IL-4-producing capacity in bone marrow-derived cells.

It has been previously reported that bone marrow progenitor cells of STAT5a/b$^{-/-}$ mice formed reduced numbers of colonies in response to IL-5, suggesting that IL-5 might depend on STAT5a/b to induce eosinophil differentiation (11). However, it is unknown whether IL-5 depends on STAT5a/b to induce IL-4-producing capacity in bone marrow-derived cells. To test this, we primed lin$^-$ bone marrow progenitor cells from STAT5a/b$^{-/-}$ mice with SCF plus IL-4 or IL-5 for 9 days. We analyzed cell numbers and compositions in colonies derived from IL-5-treated cultures. We observed that total numbers of cells derived from STAT5a/b$^{-/-}$ progenitor cells in response to IL-5 were reduced 3-fold compared with that derived from WT control (Fig. 4*A*). The percentage of eosinophils in recovered STAT5a/b$^{-/-}$ cells reduced 43% compared with that of WT mice (Fig. 4*B*). More dramatically, when we examined the IL-4-producing capacity by the recovered cells, we noted that the recovered cells from IL-5-treated culture failed to produce significant amounts of IL-4 (Fig. 4*C*). IL-4 is not known to use STAT5 for its signaling, and we thus used it as a positive control. IL-4 showed moderate reduction in its ability to drive bone marrow progenitor cells to develop into IL-4-producing cells in the absence of STAT5 ($p = 0.4$). These data suggest that, although there are synergistic effects between IL-4 and IL-5, IL-4 and IL-5 used a different signaling pathway to exert their biological functions.

The results, which show that STAT5a/b$^{-/-}$ failed to develop into Th2 cytokine-producing eosinophils in response to IL-5, prompt us to investigate the role of STAT5 in Th2 cell development. We primed naive CD4$^+$ T cells from WT and

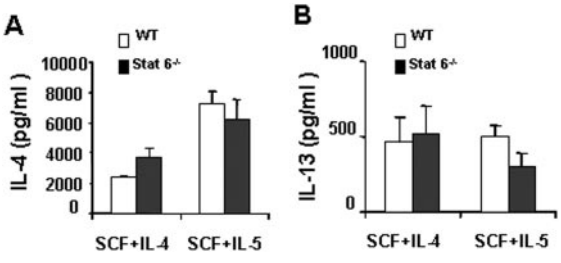

FIGURE 2. IL-4 induces differentiation of Th2-type innate cells independent of STAT6 signaling. lin$^-$ bone marrow progenitor cells from WT ($n = 5$) and STAT6$^{-/-}$ ($n = 5$) mice were cultured in the presence of SCF plus IL-4 or IL-5 for 9 days. The resultant cells were washed extensively and stimulated (1×10^6 in 1 ml) with PMA and ionomycin overnight. IL-4 and IL-13 proteins were measured by ELISA.

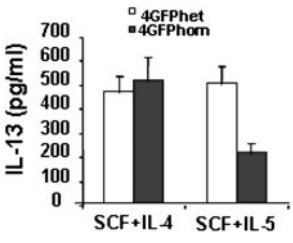

FIGURE 3. IL-5 does not depend on IL-4 to induce differentiation of IL-13-producing eosinophils. lin$^-$ bone marrow progenitor cells from 4GFP hom ($n = 2$) and 4GFP het mice ($n = 2$) were cultured in the presence of SCF plus IL-4 or IL-5 for 9 days. The resultant cells were harvested and stimulated with PMA and ionomycin overnight. IL-13 protein was measured by ELISA.

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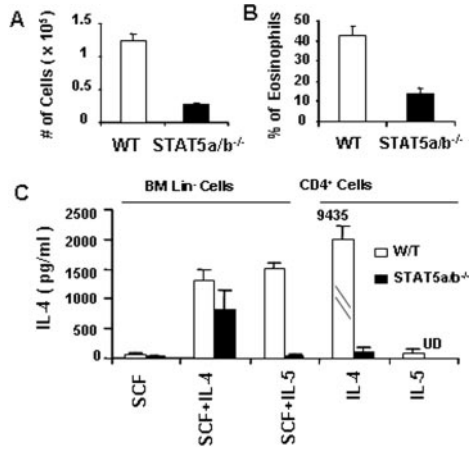

FIGURE 4. STAT5a/b is required for IL-5 to prime bone marrow progenitor cells into IL-4-producing eosinophils. *A*, lin^- bone marrow progenitor cells from C57BL/6 ($n = 4$) or STAT5a/$b^{-/-}$ ($n = 4$) mice were cultured (2×10^4) in the presence of SCF plus IL-5 for 9 days. The recovered cells were counted. Total number of cells represents recovered cells derived from 1×10^4 lin^- progenitor cells. *B*, Recovered cells were stained with May Grunwald Giemsa or toluidine blue. A total of 300 randomly selected cells were analyzed. *C*, lin^- bone marrow progenitor cells were treated as in *A*. Equal numbers of recovered cells (2×10^5 in 0.2 ml complete RPMI 1640) were stimulated with PMA and ionomycin overnight. Naive $CD4^+$ T cells were prepared from WT and STAT5a/$b^{-/-}$ mice and primed with IL-4 (5 ng/ml) or IL-5 (20 ng/ml) for 5 days. Recovered cells were stimulated (10^6 in 1 ml). IL-4 protein was measured by ELISA.

STAT5a/$b^{-/-}$ mice with IL-4 and IL-5. As expected, IL-5 failed to prime WT and STAT5a/$b^{-/-}$ $CD4^+$ T cells to develop into Th2 cells. However, IL-4 also failed to prime STAT5a/$b^{-/-}$ $CD4^+$ T cells to differentiate into Th2 cells (Fig. 4*C*). Because IL-2 is required for Th2 priming, the latter result may reflect the failure of IL-2 to signal in STAT5a/$b^{-/-}$ $CD4^+$ T cells (16).

Pretreatment with SCF, IL-3, and IL-6 can restore IL-5-induced growth and differentiation but not IL-5-induced IL-4 production in STAT5a/$b^{-/-}$ cells

Deficiency in IL-5-induced differentiation and Th2 cytokine production by STAT5a/$b^{-/-}$ eosinophils could be solely due to the inability of IL-5 to induce the mutant progenitor cells to proliferate. To examine this possibility, we pretreated bone marrow progenitor cells with a combination of SCF, IL-3, and IL-6, a potent combination that induces bone progenitor cells to proliferate (17). The pretreated cells were cultured with three different doses of IL-5 only without addition of SCF, IL-3, or IL-6 for additional 8 days. The pretreatment restored the IL-5-induced growth in STAT5a/$b^{-/-}$ progenitor cells from previous 27% to now 71–100% of normal control (Fig. 5*A*); and it also restored IL-5-induced eosinophil differentiation from previous 24.7–29.2% to now 55–75.4% of normal control dependent on the doses of IL-5 used (Fig. 5*B*). Conversely, the pretreatment failed to restore IL-4 production by STAT5a/$b^{-/-}$ eosinophils despite its enhanced IL-4 production by WT eosinophils from 4.2- to 6-fold (Fig. 5*C*). These data demonstrate that IL-5 drives development of Th2-type eosinophils via both STAT5-dependent and -independent signals. Initially, IL-5 needs STAT5 to prepare the progenitor cells to proliferate. This initial requirement for STAT5 can be replaced by other factors (i.e., SCF, IL-3, and IL-6). In the proliferating cells, IL-5 can

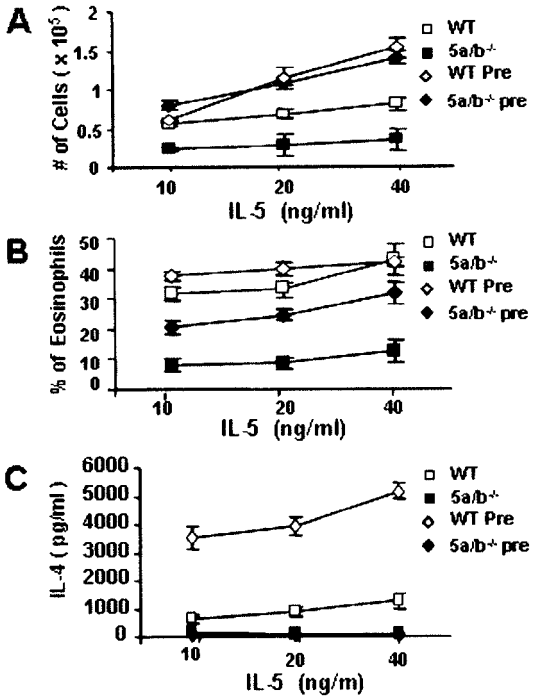

FIGURE 5. Pretreatment with SCF, IL-3, and IL-6 restores IL-5-induced growth and differentiation but not IL-5-induced IL-4 production in STAT5a/$b^{-/-}$ cells. *A*, lin^- bone marrow cells from C57BL/6 (WT) or STAT5a/$b^{-/-}$ (5a/$b^{-/-}$) mice were cultured (1×10^6/ml) in the presence of SCF (50 ng/ml), IL-3 (20 ng/ml), and IL-6 (50 ng/ml) for 1 day. Pretreated (Pre) cells or progenitor cells without pretreatment were cultured (1×10^4/dish) in the presence of IL-5 for 8 days. At the end of culture, recovered cells were analyzed for cell yields, morphology, and IL-4 production. *A*, Total cell numbers represent the number of cells recovered per dish derived from 1×10^4 lin^- cells. *B*, The percentage of eosinophils was calculated from 300 randomly selected cells. *C*, Recovered cells (10^6) in 1 ml of medium were stimulated with PMA and ionomycin overnight. IL-4 protein was measured by ELISA. These results represent two similar experiments.

maintain the cell growth and differentiation toward eosinophil lineage through STAT5-independent signals. Nonetheless, STAT5 is absolutely required for IL-5-induced IL-4 production. In another words, our data reveal differential roles for STAT5 in differentiation of eosinophils and differentiation of Th2-type eosinophils. Selective growth alone induced by IL-5 or any other factors (i.e., SCF, IL-3, and IL-6) is not sufficient to drive bone marrow progenitor cells to develop into Th2-type innate effector cells. Rather, genetic reprogramming for the *Il4* gene transcription that depends on STAT5 may be required. In a recent study conducted on the role of STAT5 in differentiation of $CD4^+$ Th2 cells, Paul and colleagues (18) showed that constitutively active STAT5a could replace IL-2 in driving naive $CD4^+$ T cells into $CD4^+$ Th2 cells. They further showed that overexpression of STAT5a caused $CD4^+$ Th2 differentiation in the absence of STAT6, suggesting that STAT5 plays a critical role in activating the *Il4* gene transcription in $CD4^+$ Th2 cells (18). Our study using STAT5a/b double deficient bone marrow progenitor cells extends their finding to bone marrow-derived cells, supporting the notion that STAT5 may play a role in transcribing the *Il4* gene in innate cells. Because of the importance of IL-4 and eosinophils in airway inflammation and asthma, we propose that this novel pathway, in which IL-4 and IL-5 drive bone marrow progenitor cells primarily into robust IL-4-producing eosinophils and inflammatory cytokines

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IL-3 and IL-6 can further enhance IL-5-induced IL-4 production, may contribute to exacerbation of airway inflammation and asthma.

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