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Changing expression of IL-3 and IL-5 receptors in cultured human eosinophils☆

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Abstract

IL-3, IL-5, and GM-CSF exert overlapping functions in eosinophils via a shared receptor β-chain, and IL-3Rα transcript expression is the weakest in blood eosinophils. We investigated the long-term regulation of surface expression of IL-3Rα. IL-3 was the most potent inducer of CD69 expression after 24-h stimulation, but not after 1-h stimulation. Expression of IL-5Rα and GM-CSFRα was significantly downregulated by culturing with their respective ligands, while IL-3Rα expression was not. IL-3 at 30 pM significantly increased IL-3Rα expression and IL-3Rα expression was also upregulated by both IL-5 and GM-CSF. In parallel with the surface protein expression, IL-3Rα mRNA was also upregulated by IL-3, IL-5, and GM-CSF. These results demonstrated that long-term culturing of eosinophils with CSFs induced a change in the potency order of CSFs, with IL-3 coming to exert the strongest effect. They thus suggest that IL-3 plays more important roles in local eosinophil activation than previously recognized.

Keywords: Eosinophils; IL-3; IL-5; GM-CSF; CD69; HLA-DR; Kinetics; Receptor

Massive eosinophil infiltration is especially prominent at the sites of inflammation associated with allergic diseases. Eosinophils promote allergic inflammation, at least in part, through the release of an array of histotoxic mediators including several granule-associated proteins [1]. Cytokines are thought to play critical roles in allergic inflammation, in view of their abilities to modulate immune responses within the allergic inflammatory cell network. Especially eosinophil-directed hemopoietins, i.e., IL-3, IL-5, and GM-CSF, have been strongly implicated in the pathogenesis of eosinophilic inflammation [2]. In addition to their induction of proliferation of progenitors of eosinophil lineage, these colony-stimulating factors (CSFs) stimulate terminally differentiated eosinophils to enhance their various biological functions, which include mediator release, adhesion molecule expression, and longevity [2].

The receptors for IL-3, IL-5, and GM-CSF all possess a heterodimeric structure having a distinct α-chain but sharing a common β-chain (βc) [3]. Each CSF binds specifically to its corresponding receptor α-chain but does not interact directly with the βc. However, βc is fully responsible for transducing intracellular signals, thereby resulting in the overlapping of biological functions exhibited by the three cytokines [3,4]. On the other hand, the expression levels of the α-chains determine the biological potency of each CSF. We recently demonstrated that the expression order of α-chain transcripts was IL-5Rα ⩾ GM-CSFRα > IL-3Rα in freshly isolated eosinophils: eosinophils express almost equivalent levels of IL-5Rα and GM-CSFRα, but IL-3Rα was the least

☆ *Abbreviations*: CSF, colony-stimulating factor; βc, common β-chain; MESF, molecules of equivalent soluble fluorochrome units.

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abundant in these cells [5]. Despite the weaker expression of the IL-3Rα transcript in eosinophils, however, we found that IL-3 exerted the most potent effect on CD69 expression after 24 h of stimulation [5]. One possible explanation for this seeming discrepancy is that culturing of eosinophils with CSFs might modulate the expression levels of the respective receptors in different fashions. In fact, recent studies by others have demonstrated that expression of IL-5Rα but not GM-CSFRα in eosinophils was strongly downregulated by culturing with the respective ligand [6]. Although an earlier study by Wang et al. [7] showed that IL-3Rα mRNA in eosinophils is upregulated by IL-3, IL-5, and GM-CSF, there has been no detailed study of the regulation of IL-3Rα focusing on surface protein expression.

A better understanding of the regulation of IL-3Rα expression in eosinophils is potentially important for establishing a therapeutic strategy for eosinophilic inflammation. Here, we demonstrate that, in contrast to IL-5Rα and GM-CSFRα, absolutely no significant decrease in IL-3Rα expression was observed after culturing with the respective ligand, whereas a certain concentration of IL-3 markedly upregulated IL-3Rα expression.

Materials and methods

Reagents. Human rIL-3 was donated by Kirin Brewery (Tokyo, Japan). Human rGM-CSF and human rIL-5 were purchased from PeproTech (London, UK). The following antibodies were purchased as indicated: PE-conjugated GM-CSFRα mAb (CD116, IgG1, clone SCO6, Coulter Immunotech, Marseille, France); PE-conjugated IL-3Rα mAb (CDw123, IgG1, clone 9F5, BD Biosciences, San Jose, CA); PE-conjugated IL-5Rα mAb (CDw125, IgG1, clone A14, BD Biosciences), recognizing both GM-CSF-bound and -unbound receptors; PE-conjugated βc mAb (CD131, IgG1, clone ICI, eBioscience, San Diego, CA, USA); PE-conjugated mouse IgG1 (Coulter Immunotech); FITC-conjugated anti-CD69 mAb (IgG1, clone FN50, eBioscience); FITC-conjugated anti-HLA-DR (IgG2a, clone L234, BD Biosciences); FITC-conjugated mouse IgG1 (Coulter Immunotech); and FITC-conjugated mouse IgG2a (PharMingen, San Diego, CA). It has been reported that prior treatment with IL-3 or IL-5 does not interfere with the analysis using anti-IL-3Rα or anti-IL-5Rα mAb, respectively [8,9].

Plasmids containing entire IL-3Rα, IL-5Rα, and GM-CSFRα sequences were kindly provided by Dr. Toshio Kitamura (University of Tokyo, Institute of Medical Science, Tokyo, Japan).

Flow cytometry of peripheral eosinophils. Eosinophils were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases. Eosinophils were purified by Percoll density gradient centrifugation followed by negative selection using anti-CD16-bound micromagnetic beads (Miltenyi BioTech, Bergisch-Gladbach, Germany) as previously described [10]. The purity and viability were consistently >99% and >95%, respectively. Eosinophils ($2–10 \times 10^4/200\,\mu l$) were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in 5% CO_2 in flat-bottomed 96-well culture plates (Iwaki, Chiba, Japan).

Expression of IL-3Rα, IL-5Rα, GM-CSFRα, and βc was analyzed using PE-conjugated mAbs (10 μg/ml) as previously described [5]. Expression of HLA-DR was analyzed using FITC-labeled mAbs (10 μg/ml). An isotype-matched mouse IgG was used as a negative control. Stained cells were analyzed using an EPICS XL SYSTEM II (Coulter, Miami, FL). The median values of fluorescence intensity were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as described previously [5]. Expression levels were calculated using the following formula: ΔMESF = (MESF of cells stained with mAb) – (MESF of cells stained with control IgG).

Real-time quantitative PCR analysis for IL-3Rα, IL-5Rα, and GM-CSFRα. Real-time quantitative PCR analysis was performed as previously described [5]. In brief, total RNA was extracted from highly purified eosinophils (purity: 99.6 ± 0.4%) using a QIAGEN RNeasy Mini Kit (Qiagen, Hilden, Germany) and the first-strand cDNA was reverse-transcribed. Real-time PCR was performed using an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The standard curve was constructed with serial 5-fold dilutions of plasmids containing the respective target sequence, as described previously [5]. The amplification efficacy ($E = 10^{-1/\text{slope}}$) was calculated from the slope of the standard curve, and the primer sets

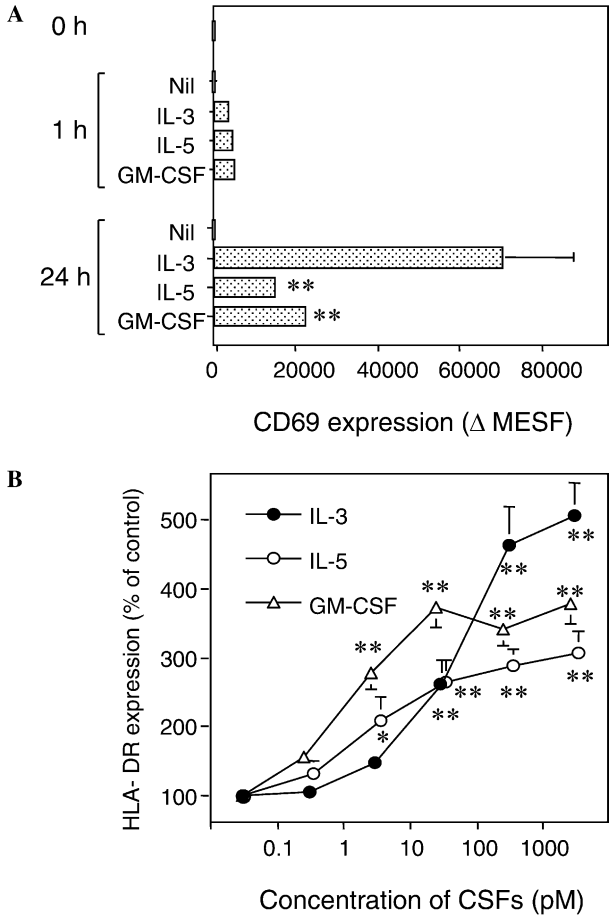

Fig. 1. Effects of CSFs on CD69 (A) and HLA-DR (B) expression in eosinophils. (A) Purified eosinophils were incubated with IL-3 (300 pM), IL-5 (300 pM), or GM-CSF (300 pM) or in medium alone (Nil) for 1 and 24 h. Then the surface CD69 expression was measured by flow cytometry. Bars represent the SEM ($n = 6$). $^{**}p < 0.01$ vs. IL-3-treated cells. (B) Purified eosinophils were incubated with serially diluted IL-3 (closed circles), IL-5 (open circles), and GM-CSF (open triangles) for 24 h. Then the HLA-DR expression was measured by flow cytometry. The values are expressed as percentages of the control expression. Control expression in the absence of CSF was 6490 ± 1705. Bars represent the SEM ($n = 6$). $^{**}p < 0.01$, $^{*}p < 0.05$, vs. Nil.

used had high efficacy between 1.89 and 1.96. The correlation coefficient of the standard curve was always >0.997.

Statistics. All data are expressed as means ± SEM. Differences between values in the in vitro experiments were analyzed by the one-way ANOVA test. When this test indicated significant differences, Fisher's protected least significant difference (PLSD) test was used to compare individual groups.

Results and discussion

Both eosinophils and basophils express all of the IL-3/IL-5/GM-CSF receptor family members, but their expression profiles are entirely different. We have previously shown that the expression order of transcripts was IL-5Rα ⩾ GM-CSFRα > IL-3Rα in eosinophils and IL-3Rα > IL-5Rα > GM-CSFRα in basophils [5]. The surface expression levels of the α-chains potentially determine the biological potency of the respective CSFs. In fact, we have observed that the potencies of CSFs on eosinophil CD11b expression after 30 min of stimulation correspond exactly with the receptor expression levels and IL-3 exerted the weakest effect [5]. However, a different situation was found in long-term stimulated cells: IL-3 induced the highest level of CD69 expression in eosinophils stimulated for 24 h [5]. Fig. 1A shows the effects of CSFs on eosinophil CD69 expression after short-term (1 h) and long-term (24 h) stimulation. It must be mentioned that IL-3 was never the strongest in inducing CD69 expression early during the stimulation. In cells stimulated for 24 h, the highest level of CD69 expression was induced by IL-3. In cells stimulated for 1 h, however, no significant difference was observed in the expression levels induced by the three CSFs. Furthermore, the strongest effect of IL-3 in long-term stimulated eosinophils was not observed merely on CD69 expression. As shown in Fig. 1B, eosinophil HLA-DR expression [11] was also upregulated by all three CSFs after 24-h stimulation, but the maximum expression level induced by IL-3 was significantly higher compared with those induced by IL-5 and GM-CSF ($p < 0.05$ and $p < 0.01$ for IL-5 and GM-CSF, respectively).

The aforementioned results revealed that not short-term but long-term culturing of eosinophils with CSFs induced a change in the potency order of CSFs, with IL-3 coming to exert the strongest effect. When IL-3 was eliminated by washing after 1 h of stimulation, a further increase in CD69 expression was profoundly suppressed (ΔMESF: 340 ± 174, 6762 ± 2028, and 72,708 ± 12,618 for 1-h-cultured, 24-h-cultured with washing, and 24-h-cultured without washing, respectively: $n = 6$), indicating that induction of CD69 expression required continuous stimulation with IL-3. Therefore, as mentioned in our previous report [5], one possible explanation for the change in the potency order of CSFs is that culturing with CSFs alters the expression levels of their respective receptors in different fashions. In the next series of experiments, we examined the surface expres-

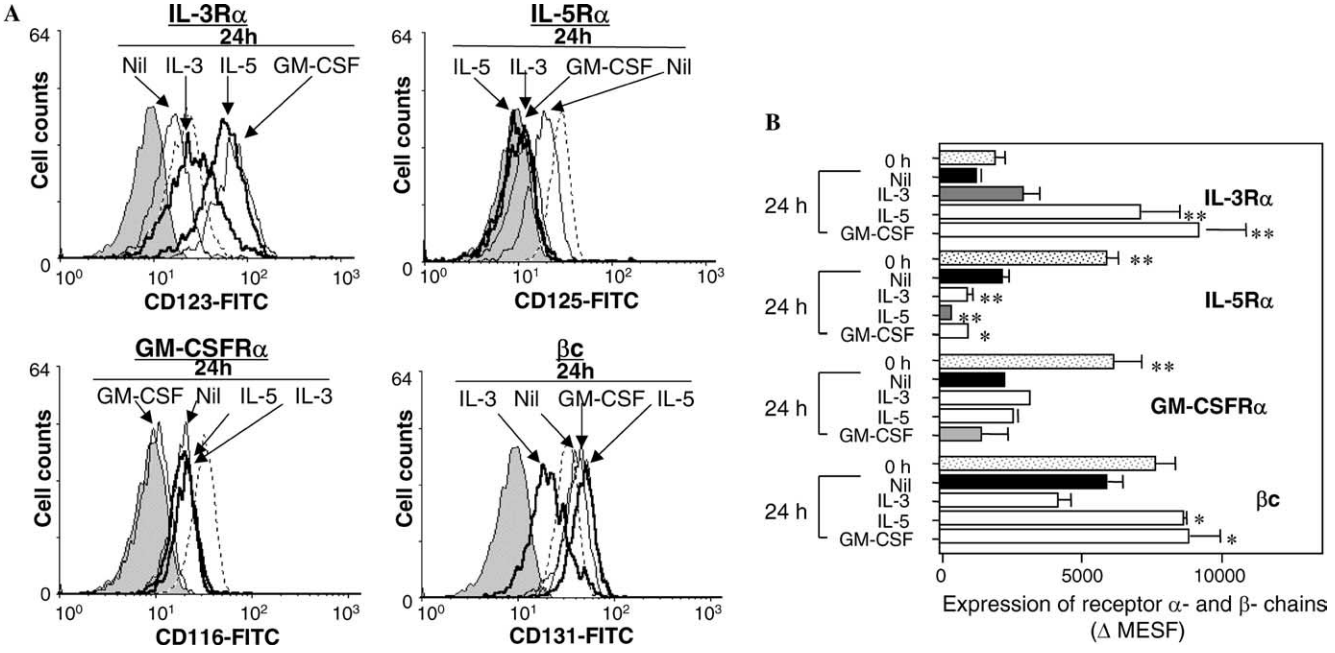

Fig. 2. Expression profiles of IL-3/IL-5/GM-CSF receptor family members by eosinophils stimulated with CSFs. Purified eosinophils were cultured with IL-3 (300 pM), IL-5 (300 pM), or GM-CSF (300 pM) or in medium alone (Nil) for 24 h, and then the surface expression levels of IL-3 Rα, IL-5Rα, GM-CSFRα, and βc were measured by flow cytometry. (A) A representative of four separate experiments is shown. The shaded area indicates the fluorescence of cells stained with PE-conjugated control mouse IgG. The dotted line indicates the fluorescence of freshly isolated cells. (B) All data are expressed as means ± SEM ($n = 4$) of MESF values. $^{**}p < 0.01$, $^{*}p < 0.05$, vs. Nil.

sion levels of IL-3Rα, IL-5Rα, GM-CSFRα, and βc on freshly isolated eosinophils and cells cultured for 24 h with 300 pM of IL-3, IL-5 or GM-CSF (Fig. 2). Consistent with earlier findings of others [6], surface IL-5Rα expression was almost completely attenuated by incubation with IL-5. In contrast to a report by others [6], surface GM-CSFRα expression was also significantly downregulated by GM-CSF. On the other hand, the surface expression level of IL-3Rα was not downregulated at all by incubation with IL-3. Furthermore, IL-3Rα expression was drastically upregulated by both IL-5 and GM-CSF: ~4- and ~5-fold increases in the surface expression were observed in cells stimulated with IL-5 and GM-CSF, respectively. On the other hand, as reported previously, surface expression of IL-5Rα was significantly downregulated by both IL-3 and GM-CSF (Fig. 2).

As shown in Fig. 3A, the effects of IL-3 on IL-3Rα expression showed a bell-shaped dose-dependent relationship. Although 300 pM of IL-3 failed to induce a significant increase in surface IL-3Rα expression, a nearly 4-fold increase in the expression was observed in cells stimulated with 30 pM of IL-3 (Fig. 3A). The bell-shaped dose-dependent curve of IL-3 strongly suggests that surface expression of IL-3Rα is regulated by a balance between de novo appearance of receptors on the cell surface and elimination of receptors via ligand-induced internalization. On the other hand, typical S-shaped dose-dependent curves with a plateau effect at 30 pM were observed for cells stimulated with either IL-5 or GM-CSF. In addition, dose-dependent inhibition of IL-5Rα expression was observed in cells treated with each of the CSFs, with plateau levels achieved at 30 pM of each CSF (Fig. 3B).

The time kinetics of surface expression of IL-3Rα and IL-5Rα are depicted in Fig. 4. No significant decrease in the level of IL-3Rα expression was observed in cells treated with 300 pM of IL-3 throughout 48 h of culturing. In cells treated with 30 pM of IL-3, a significant increase in IL-3Rα expression was observed at 8 h of stimulation ($144.3 \pm 26.1\%$ and $89.1 \pm 15.8\%$ of freshly isolated cells for cells cultured with or without IL-3, respectively, $n = 3$, $p < 0.05$). Furthermore, the expression levels of IL-3Rα were significantly increased at 24 h and maintained at high levels even at 48 h in cells stimulated with IL-5 and GM-CSF (Fig. 4A). Consistent with a previous report by others [6], the level of surface IL-5Rα expression was decreased rapidly: while eosinophil IL-5Rα expression was spontaneously decreased even in the absence of CSFs, a more rapid decrease in the expression was observed in cells treated with IL-3, IL-5 or GM-CSF. In cells treated with IL-5, a significant decrease in IL-5Rα expression was observed as early as at 1 h of stimulation, and almost complete attenuation of the expression was achieved at 4 h and sustained thereafter. Treatment with IL-3 or GM-CSF also downregulated IL-5Rα expression, albeit with slower kinetics (Fig. 4B).

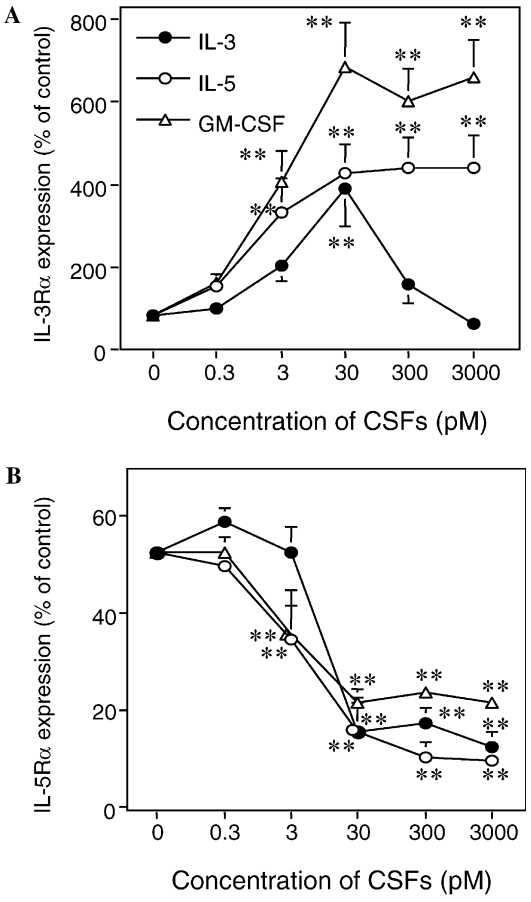

Fig. 3. Dose-dependent effects of CSFs on surface expression of IL-3Rα (A) and IL-5Rα (B) by eosinophils. Purified eosinophils were cultured with serially diluted IL-3 (closed circles), IL-5 (open circles) or GM-CSF (open triangles) for 24 h, and then the expression levels of IL-3Rα, and IL-5Rα were measured by flow cytometry. The values are expressed as percentages of the expression levels in freshly isolated cells (2393 ± 579 and 5646 ± 621 for IL-3Rα and IL-5Rα, respectively). Bars represent the SEM ($n = 4$). $^{**}p < 0.01$ vs. cells cultured for 24 h in medium alone.

When the time kinetics of mRNA expression were examined by real-time quantitative PCR, we found concordance between IL-3Rα mRNA accumulation and surface expression of the protein, indicating pretranslational regulation of surface IL-3Rα expression. As shown in Fig. 5A, stimulation with either IL-5 or GM-CSF resulted in a strong increase in mRNA of IL-3Rα which peaked at 3 h of stimulation. Treatment with IL-3 also promoted accumulation of IL-3Rα transcripts, albeit the time kinetics was rather slower compared with those by IL-5 and GM-CSF. On the other hand, as reported by others [6], accumulation of IL-5Rα mRNA was spontaneously decreased even when the cells were cultured in the absence of CSFs, and no additional reduction of the transcript was observed in cells treated with IL-3, IL-5 or GM-CSF (Fig. 5B).

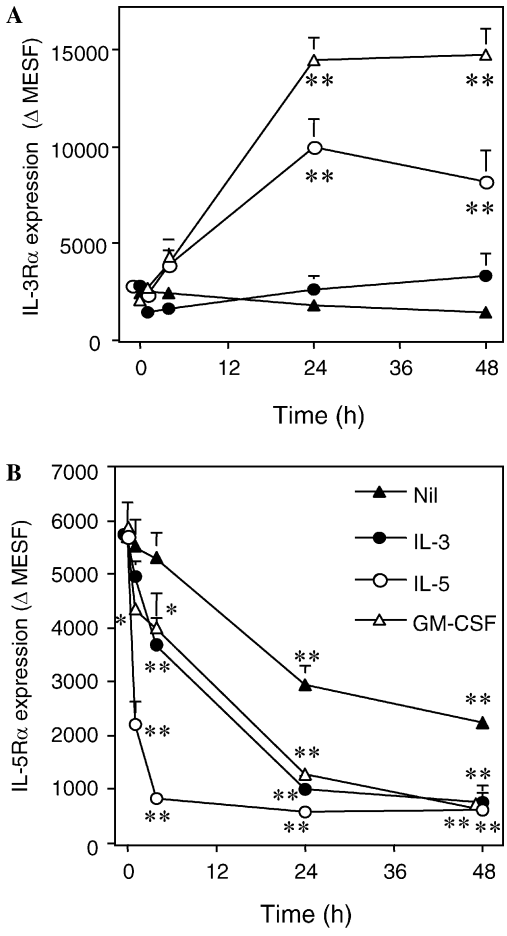

Fig. 4. Time kinetics of surface protein expression of IL-3Rα (A) and IL-5Rα (B) by eosinophils. Purified eosinophils were cultured with IL-3 (300 pM: closed circles), IL-5 (300 pM: open circles), or GM-CSF (300 pM: open triangles) or in medium alone (Nil: closed triangles). After the indicated time periods, the expression levels of IL-3Rα and IL-5Rα were measured by flow cytometry. Bars represent the SEM ($n = 4$). $^{**}p < 0.01$, $^{*}p < 0.05$, vs. Nil.

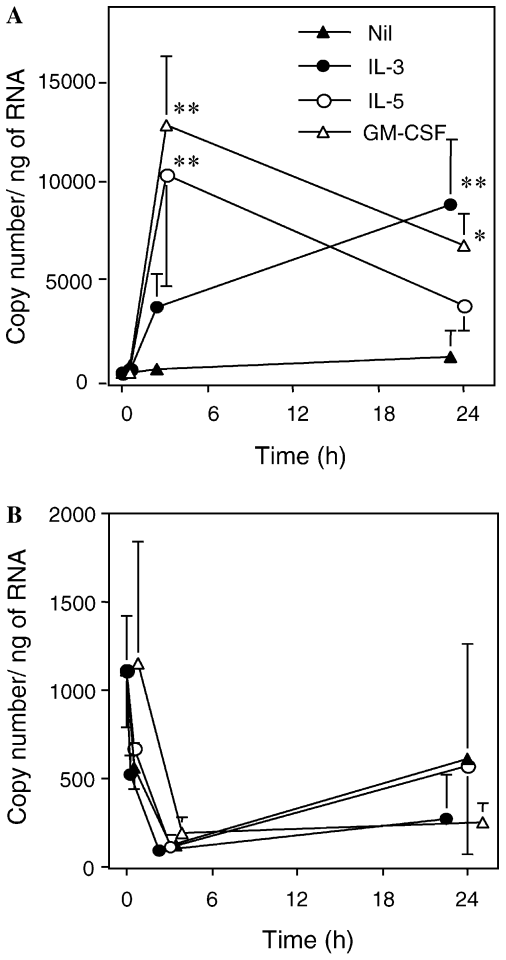

Fig. 5. Time kinetics of IL-3Rα (A) and IL-5Rα (B) mRNA expression by eosinophils. Purified eosinophils were cultured with IL-3 (300 pM: closed circles), IL-5 (300 pM: open circles), or GM-CSF (300 pM: open triangles) or in medium alone (Nil: closed triangles). Then the total cellular RNA was extracted for real-time PCR analysis. The values are expressed as copy numbers per 1 ng of RNA. Bars represent the SEM ($n = 3$). $^{**}p < 0.05$, vs. Nil.

The results presented herein clearly demonstrated uniqueness of the time kinetics of surface expression of IL-3Rα among the IL-3/IL-5/GM-CSF receptor family members. In contrast to IL-5Rα and GM-CSFRα, absolutely no significant decrease in the expression of IL-3Rα was observed after culturing with the respective ligand, but a certain concentration of IL-3 (30 pM) markedly upregulated the expression (Figs. 2–4). Sustained surface expression of IL-3Rα has functional significance: IL-3 most potently affected the expression of CD69 and HLA-DR in long-term stimulated eosinophils (Fig. 1). We previously demonstrated that the IL-3Rα transcript is the least abundant in freshly isolated eosinophils, its level being 50- to 100-fold lower than the level in basophils [5]. These expression profiles of IL-3Rα may result in too-simple conclusion that IL-3 is the key CSF for basophils but not for eosinophils. However, our present results indicate the need for reconsideration of the roles of IL-3 on eosinophils: IL-3 may play a major role in eosinophil activation under certain conditions.

Along with the progression of allergic responses, circulating eosinophils migrate to the inflammatory sites with possible consequent contribution to the pathogenesis of local tissue damage via releasing diverse pro-inflammatory mediators [12]. Although eosinophils have long been strongly looked upon as a main target in the therapeutic strategy for bronchial asthma [13,14], a recent clinical trial of anti-IL-5 has cast doubt on the central position of IL-5 and eosinophils in asthma [15]. Although the precise role of eosinophils in the pathogenesis of asthma has again become a matter of controversy, one important aspect we should keep in mind is that targeting IL-5 may not be equivalent to targeting eosinophils. As indicated in Figs. 2–4, CSF-exposed eosinophils showed apparent downregulation of surface IL-5Rα expression: this in vitro mechanism seems to

explain why locally accumulated eosinophils demonstrate decreased expression of surface IL-5Rα in vivo [9]. Our results further suggest that tissue eosinophils may prefer IL-3 as a functional regulator rather than IL-5, via selective upregulation of IL-3Rα. Thus, future in vivo studies focusing on IL-3 expression in inflamed tissues and IL-3Rα expression in tissue eosinophils will be important for assessing the pathogenesis of asthma, and especially for clarifying the role of this cytokine.

During preparation of this manuscript, Gregory et al. [16] reported findings basically similar to some of the data reported herein. Their studies and ours collectively suggest that IL-5, which is clearly involved in the pathogenesis of blood eosinophilia [17,18], may not be central for regulating the biological functions of tissue eosinophils. Moreover, our present data, in combination with our recent paper demonstrating that tissue basophils possess surface CD69 which can be induced in vitro exclusively by IL-3 [19], strongly suggest that IL-3 may play more important roles in the pathogenesis of airway allergy than previously recognized.

Acknowledgments

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