American Journal of Pathology, Vol. 144, No. 1, January 1994

Effect of Interleukin-4 on the Synthesis of the Third Component of Complement by Pulmonary Epithelial Cells

Karlyle K. Christian-Ritter, Laura D. Hill, Eric B. Hoie, and Terence L. Zach

From the Joint Division of Newborn Medicine, Departments of Pediatrics, Creighton University and University of Nebraska Medical Center, Omaha, Nebraska

Complement activation in the lung is important in a variety of physiological and pathological conditions. The third component of complement, C3, is the pivotal constituent of the complement cascade. C3 is produced in the lung by several cell types including pulmonary epithelial cells. Because pulmonary epithelial cells and T lymphocytes may interact within the lung to regulate local immune responses, we examined the effect of a T lymphocyte-derived cytokine, interleukin-4 (IL-4) on C3 production by A549 human pulmonary epithelial cells. Treatment of A549 cells with IL-4 increased C3 production in a time- and dose-dependent fashion. Concentrations of IL-4 ≥ 0.1 ng/ml significantly increased C3 production. Maximal increase in C3 synthesis occurred after stimulation of A549 cells with IL-4 (10 ng/ml) for 3 days. Preincubation of IL-4 with a neutralizing anti-human IL-4 antibody inhibited IL-4's effect on C3 production. The relative abundance of C3 messenger RNA levels in A549 cells increased following IL-4 treatment, indicating that IL-4's effects on C3 production were pretranslational. Intercellular communication between T lymphocytes and pulmonary epithelial cells via cytokines such as IL-4 may be important in the local regulation of C3 gene expression during the inflammatory response. (Am J Pathol 1994, 144:171–176)

The third component of complement, C3, is a central mediator of the inflammatory response. C3 occupies a pivotal position in the complement cascade being situated at the junction of the classical and alternative pathways of complement activation. Upon activation by either pathway, C3 is cleaved to C3a and C3b. C3a is an anaphylatoxin, and C3b binds to the bacterial surface and initiates phagocytosis mediated by complement receptors on neutrophils and macrophages. Cleavage of C3 also results in the formation of the C5 convertase, promoting the assembly of the membrane attack complex, C5b-9, and the liberation of the neutrophil chemotaxin, C5a.

The hepatocyte is the primary source of C3 that circulates through the bloodstream. However, local production of C3 in the lung may be important in host defense and in pulmonary inflammation. The types of pulmonary cells that have been identified as capable of synthesizing C3 include alveolar macrophages, pulmonary fibroblasts, and type II pulmonary epithelial cells.[1–3] Recent studies have suggested that the type II pulmonary epithelial cell is the principal pulmonary source of C3.[2,3]

The biological messengers that regulate the synthesis of C3 by pulmonary epithelial cells are not fully known. Activated T cells in the lung secrete lymphokines that could act in a paracrine fashion to regulate pulmonary epithelial cell activity. We examined the effect of one such T cell-derived cytokine, interleukin-4 (IL-4), on C3 production by the human pulmonary epithelial cell line, A549. We report that IL-4 increases C3 synthesis by A549 cells. We demonstrate that the effect of IL-4 on C3 production can be neutralized by preincubation with an anti-human IL-4 antibody, and we provide evidence that IL-4's effects on C3 production are pretranslational.

Materials and Methods

A549 cells were obtained from American Type Culture Collection (Rockville, MD). Recombinant hu-

Supported in part by an American Lung Association (ALA) research grant, an Edna Ittner Pediatric research grant, and a University of Nebraska Medical Center seed grant. Dr. Zach is a recipient of the ALA Edward Livingston Trudeau Scholar Award.

Accepted for publication August 31, 1993.

Address reprint requests to Dr. Terence L. Zach, 3001 Douglas, North Tower 7th Floor, Omaha, NE 68131.

man IL-4 was purchased from Genzyme Biochemicals Ltd. (Cambridge, MA). The IL-4 had an approximate specific activity of 1×10^8 units/mg and was diluted in culture media containing 10% fetal calf serum according to the manufacturer's instructions. Goat antibody (immunoglobulin G) to human C3 was obtained from Atlantic Antibodies (Scarborough, ME), peroxidase-conjugated goat antibody (immunoglobulin G) to human C3 was obtained from Cappel Laboratories (Malvern, PA), and neutralizing antibodies (immunoglobulin G) to human IL-4 and human IL-1β were obtained from R & D Systems (Minneapolis, MN). Purified human C3 was kindly provided by Dr. Margaret K. Hostetter (Minneapolis, MN).

Cell Culture

A549 cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 1% vitamins, 4 mmol/L L-glutamine, 1% nonessential amino acids, and 50 µg/ml gentamicin. The cells were grown in 150-cm^2 tissue culture flasks, incubated at 37 C with 5% CO_2 in air, and supplemented with fresh medium every 2 to 3 days.

For quantitation of C3 production, A549 cells were seeded at a concentration of 5×10^5 cells/flask in 4 ml media in 25-cm^2 tissue culture flasks. Cells were allowed to adhere overnight. The media was changed and IL-4 was added on what was considered day 0. A 0.5-ml aliquot of cell supernatant was removed daily and replaced with 0.5 ml of fresh media. Supernatant samples were stored at −20 C for later C3 quantitation.

For analysis of C3 production in the presence of IL-4 and neutralizing antibodies, A549 cells were seeded at a concentration of 5×10^4 cells/well in 24-well tissue culture plates. Cells were allowed to adhere overnight and then the media was changed. IL-4 was preincubated with either anti-IL-4 or anti-IL-1β for 2 hours at 37 C. IL-4 with or without neutralizing antibody was added on day 0. Supernatants were harvested on day 3 and stored at −20 C for later C3 quantitation. For analysis of C3 messenger (m)RNA expression, cells were seeded at a concentration of 3 to 5×10^6 cells/flask in 20 ml media in 75-cm^2 tissue culture flasks. IL-4 was added to the cell cultures as described.

Measurement of C3 concentrations by Enzyme-Linked Immunosorbent Assay (ELISA)

C3 concentration in A549 supernatants was measured by sandwich Enzyme-Linked Immunosorbent Assay.[4] Purified human C3 was used for calibration of the standard curve. Wells of a 96-well microtiter plate were coated with 0.1 ml of a 1:400 dilution of goat antibody to human C3 in carbonate/bicarbonate buffer (pH 9.6), incubated overnight at 4 C, and washed three times with 0.05% Tween 20 in phosphate-buffered saline. A549 supernatants were added to duplicate wells in a volume of 0.1 ml and incubated for 1 hour at 37 C. After three washes, 0.1 ml of a 1:800 dilution of peroxidase-conjugated goat antibody to human C3 was added to each well and incubated for 1 hour at 37 C. The developing reagent consisted of 0.4 mg/ml of o-phenylenediamine in 0.2 mol/L Na_2HPO_4 (pH 5.0 with 0.1 mol/L citric acid), containing 0.4 µl 35% H_2O_2 per ml. Color was developed for approximately 10 minutes, and absorbance measured at 492 nm.

Northern Blot Analysis

Polyadenylated mRNA was prepared from A549 cells by a modification of the method of Badley et al.[5] A549 cell monolayers were washed free of media with cold phosphate-buffered saline, detached with 0.05% trypsin/0.5 mmol/L ethylenediaminetetraacetic acid, and lysed by a lysis buffer composed of 400 µg/ml proteinase K, 2% sodium dodecyl sulphate (SDS), 1.5 mmol/L $MgCl_2$, 0.2 mol/L NaCl, and 0.2 mol/L Tris HCl, pH 7.5. The suspension was homogenized with a Dounce homogenizer and incubated with gentle shaking at 45 C for 2 hours. The concentration of NaCl of the lysate was adjusted to 0.5 mol/L with 5 mol/L NaCl stock solution. The lysate was mixed and added to 100 mg of hydrated oligo (dT) cellulose (Boehringer Mannheim Biochemicals, Indianapolis, IN), which was equilibrated with a binding buffer composed of 0.5 mol/L NaCl and 0.01 mol/L Tris HCl, pH 7.5. The mixture was gently agitated for 20 minutes at room temperature, then centrifuged at 1,000 rpm for 4 minutes, and the pellet was washed several times with binding buffer until the supernatant was clear. The oligo (dT) cellulose was transferred to siliconized disposable columns and washed further until the optical density of the eluate was less than 0.05 at 260 nm. The polyadenylated mRNA was eluted from the column with RNAse-free H_2O. The mRNA was precipitated with 2 vol 95% ethanol in the presence of 0.1 vol 3 mol/L sodium acetate at −80 C for 1 hour. Following a wash in 0.5 ml 70% ethanol and drying of the sample, the mRNA was solubilized in RNAse-free H_2O and quantitated spectrophotometrically. Aliquots of mRNA (3 to 5 µg) were denatured in 50% formamide, 17% formaldehyde, 7%

glycerol, 20 mmol/L morpholine-propanesulfonic acid, pH 7.0, 5 mmol/L sodium acetate, 1 mmol/L ethylenediaminetetraacetic acid at 65 C for 15 minutes then cooled on ice for 3 minutes. mRNA was fractionated by electrophoresis in a 1% agarose gel containing 6% formaldehyde, 20 mmol/L morpholine-propanesulfonic acid, 5 mmol/L sodium acetate, and 1 mmol/L ethylenediaminetetraacetic acid for 16 hours at 55V. Following electrophoresis, the gels were washed in RNAse-free H_2O, then 10× standard saline citrate (SSC) (1× SSC is 0.015 mol/L sodium citrate and 0.15 mol/L NaCl) for 15 minutes and transferred overnight to nylon membranes (Schleicher and Schuell Inc., Keene, NH) in 10× SSC. The filters were baked at 80 C for 2 hours, then prehybridized at 42 C for 4 hours in 20% formamide, 5× SSC, 0.1% sodium dodecyl sulphate, 5× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA. Filters were hybridized 16 hours with the 2.4- and 1.8-kb fragments of the human C3 complementary DNA probe, pHLC3.11 (American Type Culture Collection).[6] These C3 complementary DNA insert fragments encompass the coding sequence for approximately 90% of C3 measured from the COOH end and include the COOH region of the β chain and all of the coding sequences for the α chain.[6] Probes were radiolabeled with [^{32}P]CTP to a specific activity of 1 to 5 × 10^5 cpm/ng DNA by random sequence oligonucleotide priming (Boehringer Mannheim Biochemicals). Blots were sequentially washed in 2× SSC, 0.1% SDS at room temperature then 0.1× SSC, 0.1% SDS at 60 C, and autoradiographed to Kodak XAR film at −70 C. Blots were reprobed with [^{32}P]CTP labeled human tubulin complementary DNA (American Type Culture Collection).

Statistical Analysis

The two-tailed Student's *t* test, analysis of normal variance, and linear regression analysis for determination of the coefficient of correlation were used for statistical analysis. Results are expressed as mean ± SEM and $P \leq 0.05$ was considered statistically significant.

Results

Effect of IL-4 on C3 Production

A549 cells were cultured in the presence of IL-4 at a concentration of 10 ng/ml for 3 days. Supernatants were collected daily for C3 quantitation by ELISA. As shown in Figure 1, C3 production by unstimulated A549 cells increased gradually with time until day 3 in culture (57.5 ± 7.2 ng/ml). The addition of IL-4 significantly increased C3 production on days 1, 2, and 3 versus unstimulated control A549 cells. Cell viability decreased beyond day 3 in culture in both control and IL-4-treated cells.

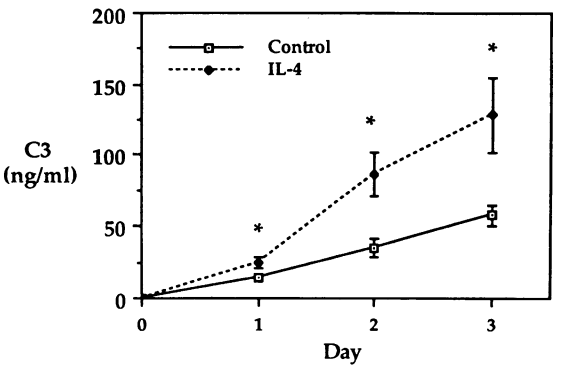

Figure 1. *Kinetics of C3 production by A549 cells in the presence or absence of IL-4 (10 ng/ml). Supernatants were harvested on days 1 to 3, and C3 concentrations were measured by ELISA. Values represent mean ± SEM (*P < 0.05 IL-4 versus control, n = 10).*

The addition of IL-4-enhanced C3 production by A549 cells in a dose-dependent manner (Figure 2). IL-4 at concentrations ≥0.1 ng/ml significantly increased C3 production. Maximal increase in C3 production occurred following stimulation of A549 cells with IL-4 at a concentration of 10 ng/ml and was 245% greater than unstimulated control cells.

To confirm that the regulation of C3 production in A549 cells is a specific property of IL-4, antibody blocking experiments were conducted. Preincubation of IL-4 (1 ng/ml) with anti-IL-4 antibody (10 μg/ml) neutralized IL-4's enhancement on C3 production (Figure 3). To determine whether the anti-IL-4's inhibitory effects were due to nonspecific effects, we examined the effects of control antibody of the

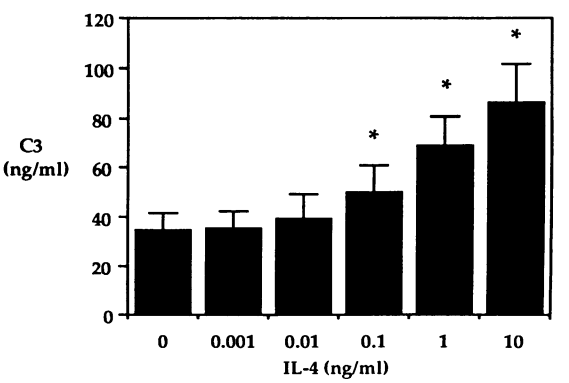

Figure 2. *Dose-response of C3 production by IL-4-treated A549 cells. Values represent C3 concentration in supernatants harvested on day 3 of culture and are reported as mean ± SEM (*P < 0.05 IL-4 versus control, n = 9).*

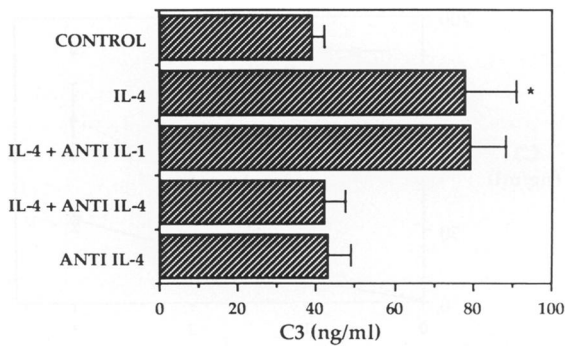

Figure 3. *Effect of neutralizing antibodies on C3 production by A549 cells. Values represent C3 concentration in supernatants harvested on day 3 of culture and are reported as mean ± SEM* (*P < *0.05 IL-4 versus IL-4 + anti-IL-4,* n = 5).

same isotype. Preincubation of IL-4 (1 ng/ml) with anti-IL-1β antibody (10 μg/ml) did not alter C3 production as compared to IL-4-stimulated cells. A549 cells treated with anti-IL-4 antibody alone had similar C3 production as untreated control cells (Figure 3).

Effect of IL-4 on C3 mRNA Levels

A549 cells were stimulated with IL-4 at a concentration of 1 ng/ml and polyadenylated mRNA was isolated at varying times. As demonstrated in Figure 4, unstimulated A549 cells constitutively express low levels of C3 mRNA as detected by Northern blot analysis. The 5.2-kb size of the C3 transcript in unstimulated and IL-4-stimulated A549 cells is comparable to the size of C3 mRNA that has been reported in other tissues.[7,8] IL-4 increased the abundance of steady state C3 mRNA levels in A549 cells.

Discussion

The complement cascade plays a central role in host defense and in the inflammatory response. C3 is considered the keystone of the complement cascade because it links the classical and alternative pathways of complement activation. Cleavage of C3 by either pathway causes the release of the anaphylatoxin C3a, leads to the deposition of C3b on the bacterial surface, and culminates in the assembly of the membrane attack complex C5b-9.

Although the liver is the primary source of circulating C3, local sources of C3 are undoubtedly important at sites of inflammation that are sequestered from the bloodstream and at portals of entry for invading microorganisms. Several cell types within

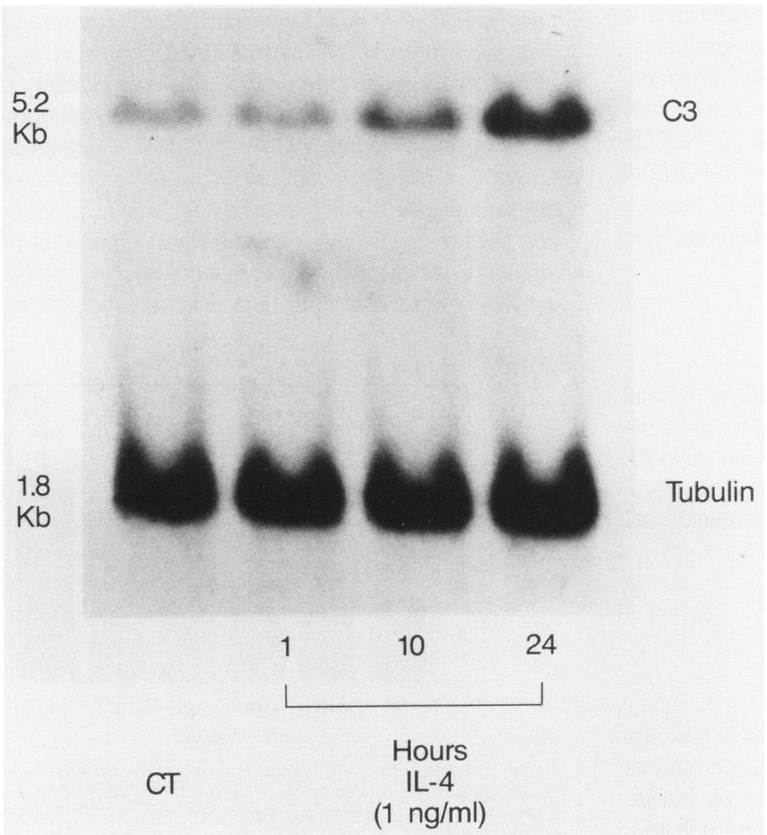

Figure 4. *Effect of IL-4 on steady-state C3 mRNA levels. Polyadenylated mRNA was isolated from A549 cells at the indicated times after the addition of IL-4 (1 ng/ml). RNA was electrophoresed through 1% agarose, 6% formaldehyde gel, transferred to nylon membranes, hybridized with a ^{32}P-labeled human C3 cDNA probe and reprobed with a ^{32}P-labeled human tubulin complementary DNA probe.*

the lung are known to synthesize and secrete C3, including alveolar macrophages and pulmonary fibroblasts.[1,3] Only recently have pulmonary epithelial cells been shown to synthesize C3.[2,3] Therefore, the spectrum of activity of pulmonary epithelial cells has been expanded to include a potential role in inflammation and host defense.

The biological messengers that regulate C3 production by pulmonary epithelial cells have not been fully elucidated. The A549 cell line has been used as a prototype to study factors that regulate C3 synthesis by pulmonary epithelial cells.[3,4,9,10] Previous investigators have shown that these cells contain unique cytoplasmic multilamellar bodies that are characteristic of type II pulmonary epithelial cells.[3] Rothman and coworkers have demonstrated that cytokines such as IL-1 and IL-2 increase C3 production by A549 cells.[6]

In the present study, we identify an additional cytokine that alters C3 production by A549 cells. Treatment of A549 cells with IL-4 increased C3 secretion in a time- and dose-dependent fashion (Figures 1 and 2). We report that preincubation of IL-4 with an anti-IL-4 antibody neutralized IL-4's enhancement of C3 synthesis (Figure 3). In addition, treatment of A549 cells with IL-4 increased the relative abundance of C3 mRNA, indicating that IL-4's effects on C3 production were pretranslational (Figure 4).

IL-4 is primarily an activated T cell-derived cytokine. IL-4 was originally identified as a factor that induced proliferation and maturation of B cells.[11,12] IL-4 is now known to have a number of diverse functions on a variety of cell types, including T cells,[13] hematopoietic cells,[14] endothelial cells,[15] and epithelial cells.[16] The functions of IL-4 can be characterized as both pro- and anti-inflammatory, depending upon the cell type and conditions studied. Examples of IL-4's pro-inflammatory properties include IL-4 augmentation of T cell proliferation,[13] IL-4 up-regulation of colony stimulating factor and tissue plasminogen activator production by monocytes and macrophages.[14,17] Among the anti-inflammatory properties of IL-4 are IL-4 inhibition of IL-1, tumor necrosis factor-α, and IL-6 synthesis and IL-4 suppression of IL-8 mRNA expression by lipopolysaccharide-stimulated monocytes.[18,19]

Little is known about the effects of IL-4 on complement gene expression. Littman and coworkers reported that IL-4 increases C2 synthesis by human monocytes and a monocytic cell line.[20] Katz and Strunk have demonstrated that IL-4 abrogates the stimulatory effect of tumor necrosis factor on complement factor B synthesis by skin fibroblasts.[21] These investigators have also reported that IL-4 does not alter the synthesis of C3 by skin fibroblasts.[21]

In summary, we have shown that recombinant human IL-4 increases C3 production by a human pulmonary epithelial cell line, designated A549. Although we provide evidence that the mechanism of IL-4's effects on C3 production are pretranslational, future studies will be necessary to determine the exact molecular mechanism of IL-4's effects and to determine whether IL-4 alters C3 gene expression in primary cultures of type II cells.

Acknowledgments

The authors thank Joan Spencer and Joe Torchia for excellent technical assistance.

References

1. Cole FS, Matthews WJ, Rossing TH, Gash DJ, Lichtenberg NA, Pennington JE: Complement biosynthesis by human bronchoalveolar macrophages. Clin Immunol Immunopathol 1983, 27:153–159
2. Strunk RC, Eidlen DM, Mason RJ: Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. J Clin Invest 1988, 81:1419–1426
3. Rothman BL, Merrow M, Bamba M, Kennedy T, Kreutzer DL: Biosynthesis of the third and fifth complement components by isolated human lung cells. Am Rev Resp Dis 1989, 139:212–220
4. Zach TL, Hill LD, Herrman VA, Leuschen MP, Hostetter MK: Effect of glucocorticoids on C3 gene expression by the A549 human pulmonary epithelial cell line. J Immunol 1992, 148:3964–3969
5. Rothman BL, Merrow M, Despins A, Kennedy T, Kreutzer DL: Effect of lipopolysaccharide on C3 and C5 production by human lung cells. J Immunol 1989, 143:196–202
6. Rothman BL, Despins AW, Kreutzer DL: Cytokine regulation of C3 and C5 by the human type II pneumocyte cell line, A549. J Immunol 1990, 145:592–598
7. Badley JE, Bishop GA, St. John T, Frelinger JA: A simple, rapid method for the purification of poly A+ RNA. Biotechniques 1988, 6:114–116
8. deBruijn MHL, Fey GH: Human complement component C3: cDNA coding sequence and derived primary structure. Proc Natl Acad Sci USA 1985, 82:708–712
9. Sato T, Hong MH, Jin CH, Ishimi Y, Udagawa N, Shinki T, Abe E, Suda T: The specific production of the third component of complement by osteoblastic cells treated with 1 alpha, 25-dihydroxyvitamin D3. FEBS Lett 1991, 285:21–24
10. Sundstrom SA, Komm BS, Ponce-de-Leon H, Yi Z, Teuscher C, Lyttle CR: Estrogen regulation of tissue-specific expression of complement C3. J Biol Chem 1989, 264:16941–16947

11. Howard M, Farrar J, Hilfiker M, Johnson B, Takatsu K, Hamaoka T, Paul WE: Identification of a T cell-derived B cell growth factor distinct from interleukin 2. J Exp Med 1982, 155:914–923
12. Rabin EM, Ohara J, Paul WE: B cell stimulatory factor (BSF-1) activates resting B cells. Proc Natl Acad Sci USA 1985, 82:2935–2939
13. Spits H, Yssel H, Takebe Y, Arai N, Yokota T, Lee F, Arai K, Banchereau J, de Vries J: Recombinant interleukin 4 promotes growth of human T cells. J Immunol 1987, 139:1142–1147
14. Hart PH, Burgess DR, Vitti GF, Hamilton JA: Interleukin-4 stimulates human monocytes to produce tissue-type plasminogen activator. Blood 1989, 74: 1222–1225
15. Thornhill MH, Haskard DO: IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-γ. J Immunol 1990, 145:865–872
16. Phillips JO, Everson MP, Moldoveanu Z, Lue C, Mestecky J: Synergistic effect of IL-4 and IFN-gamma on the expression of polymeric Ig receptor (secretory component) and IgA binding by human epithelial cells. J Immunol 1990, 145:1740–1744
17. Weiser M, Bonifer R, Oster W, Lindemann A, Mertelsmann R, Herrmann F: Interleukin-4 induces secretion of CSF for granulocytes and CSF for macrophages by peripheral blood monocytes. Blood 1989, 73:1105–1108
18. te Velde AA, Huijbens RJF, Heije K, de Vries JE, Figdor CG: Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. Blood 1990, 76:1392–1397
19. Standiford TJ, Strieter RM, Kasahara K, Kunkel SL: Disparate regulation of interleukin 8 gene expression from blood monocytes, endothelial cells, and fibroblasts by interleukin 4. Biochem Biophys Res Comm 1990, 171:531–536
20. Littman BH, Dastvan FF, Carlson PL, Sanders KM: Regulation of monocyte/macrophage C2 production and HLA-DR expression by IL-4 (BSF-1) and IFN-gamma. J Immunol 1989, 142:520–525
21. Katz Y, Strunk RC: Enhanced synthesis of factor B induced by tumor necrosis factor in human skin fibroblasts is decreased by IL-4. J Immunol 1990, 144: 4675–4680