Interleukin-6⁻¹⁷⁴ and Tumor Necrosis Factor α ⁻³⁰⁸ Polymorphisms Enhance Cytokine Production by Human Macrophages Exposed to Respiratory Viruses

Janak A. Patel,¹ Sangeeta Nair,^{1,*} Eliana E. Ochoa,^{2,†} Ruksana Huda,²
Norbert J. Roberts, Jr.,² and Tasnee Chonmaitree¹

Interleukin-6⁻¹⁷⁴ (IL-6⁻¹⁷⁴) and tumor necrosis factor α ⁻³⁰⁸ (TNF α ⁻³⁰⁸) are high-cytokine-producing genotypes that are known to increase the susceptibility to infectious diseases, but their influence on cytokine production induced by respiratory viruses is unknown. We exposed human monocyte-derived macrophages from IL-6⁻¹⁷⁴, TNF α ⁻³⁰⁸, and normal genotype donors to different respiratory viruses. Respiratory syncytial virus (RSV) stimulation was associated with higher IL-6 concentrations in IL-6⁻¹⁷⁴ (G/C or C/C) donors than in normal (G/G) donors ($P=0.015$); 2 of 7 (29%) polymorphic donors were poor responders compared with 6 of 7 (86%) normal donors ($P=0.002$). Adenovirus, influenza virus, and RSV stimulations were associated with higher TNF α concentrations in TNF α ⁻³⁰⁸ (G/A or A/A) donors than in normal (G/G) donors ($P=0.03$, <0.01 , <0.01). A similar trend was seen with rhinovirus stimulation, but this was not significant. These results show that IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ gene polymorphisms lead to enhanced production of the respective cytokines when exposed to specific respiratory viruses. This, in turn, may influence the susceptibility to, severity of, and recovery from respiratory virus infections, or influence the immune response to and reactogenicity of viral vaccines.

INTERLEUKIN-6 (IL-6) AND TUMOR NECROSIS FACTOR α (TNF α) are among the key acute-phase cytokines that are involved in inflammation due to a variety of infections. Single-nucleotide polymorphisms (SNPs) of the regulatory elements of these acute-phase cytokine genes, specifically IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸, are known to enhance production of the respective cytokines, which in turn can predispose to, or exacerbate the severity of, a wide variety of infectious diseases (Knight 2001).

Viral upper respiratory infection (vURI) is an important predisposing factor for the development of acute otitis media (AOM) (Heikkinen and Chonmaitree 2003). The pathogenesis of virus-induced AOM is complex, involving host immunogenetics, virus-bacterial interactions, and other environmental factors. We have previously reported that children with IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ polymorphic genotypes have increased susceptibility to recurrent AOM (Patel and others 2006). It is possible that these specific cytokine genotypes lead to enhanced production of IL-6 and TNF- α in the nasopharynx during the preceding vURI episode; the higher degree of inflammation may promote more

severe Eustachian tube abnormality and increase the risk for AOM development.

The IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ polymorphic genotypes may also be associated with other complications of respiratory virus infections. For example, these two polymorphic genotypes are associated with the increased risk and severity of bronchiolitis associated with respiratory syncytial virus (RSV) infection (Gentile and others 2003; Puthothu and others 2009). IL-6⁻¹⁷⁴ genotype may also influence the immunogenicity and reactogenicity of influenza virus vaccines (Poland and others 2008). However, there is no published study on the direct influence of IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ genotypes on cytokine production by the host antigen-presenting cells (APCs) exposed to respiratory viruses. APCs such as monocytes and macrophages orchestrate the crucial pathogen-specific T- and B-cell responses.

In the present study, we used an *in vitro* model of monocyte-derived macrophages from donors with IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ polymorphic genotypes to evaluate the production of respective cytokines induced by respiratory viruses. We chose RSV, rhinovirus, adenovirus, and influenza

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¹Division of Pediatric Infectious Disease and Immunology, Department of Pediatrics and ²Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas.

*Present address: Meharry Medical College, Nashville, Tennessee.

†This author is in private medical practice in McAllen, Texas.

virus as stimuli because they are among the commonest cause of vURI. Results of this study may provide better understanding of the pathogenesis of vURI as well as the immunogenicity and reactogenicity of respiratory viral vaccines.

DNA was extracted from the buccal epithelial cells of adult volunteers for IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ genotype analysis by restriction fragment length polymorphism as described previously (Galbraith and others 1999). Briefly, polymerase chain reaction (PCR) was performed on the extracted DNA with the use of respective cytokine primer sets that spanned the SNP sites. The resultant PCR products were digested with polymorphic site-specific enzymes, namely, *Nco*I for TNF α ⁻³⁰⁸ and *Hsp*92II for IL-6⁻¹⁷⁴. All genotypes that were identified by PCR and restriction fragment length polymorphism were confirmed by DNA sequencing. The study of polymorphisms yielded 3 genotypes for each cytokine: homozygous "normal" (low-cytokine-producing) genotype, and homozygous and heterozygous polymorphic (high-cytokine-producing) genotypes. Donors were consid-

ered to be polymorphic if they had either homozygous or heterozygous SNP.

Overall, 31 donors were screened for genotype; 14 donors were selected who had either IL-6⁻¹⁷⁴ ($n=7$) or TNF α ⁻³⁰⁸ ($n=7$) polymorphic genotype. The details of the medical history of the donors were unknown. The present study was approved by the local institutional ethics review board.

Peripheral blood was collected by venipuncture in ethylenediaminetetraacetic acid-containing vials. The donors were healthy and not on any immunomodulatory medications at the time of blood collection. Mononuclear cells were separated using Lymphocyte Separation Medium (Lymphosep[®]; MP Biomedicals, Solon, OH) according to the manufacturer's instructions. The mononuclear cells were further purified using the Dynabeads MyPure Monocyte Kit 2 (Invitrogen, Carlsbad, CA), which yielded more than 95% purity for monocytes. The purified monocytes were resuspended in serum-free RPMI media, and aliquots of 1×10^5 cells per 100 μ L were placed into 96-well plates and incubated overnight at 37°C. The overnight culture of the

FIG. 1. IL-6 and TNF α concentrations in the supernatant fluids of macrophage cultures incubated with various stimuli for 3 and 6 h. Values are derived from macrophages of 7 donors in each group with IL-6⁻¹⁷⁴ or TNF α ⁻³⁰⁸ polymorphic (black bars) or the respective normal genotype (white bars). Each donor's experiment was performed in triplicate. The final cytokine concentrations in the supernatant were calculated after subtracting the baseline value of cytokines present in the virus inoculum. Virus multiplicity of infection = 3; 1×10^5 macrophages/well; final volume = 200 μ L. Only significant P values are shown. The error bars represent 1 SD. IL-6, interleukin-6; TNF α , tumor necrosis factor α ; LPS, lipopolysaccharide; RSV, respiratory syncytial virus; SD, standard deviation.

monocytes yielded an early differentiated macrophage phenotype. Viruses were then added to the cells as described below.

Virus pools were prepared using standard tissue culture methods. The final concentrations of the viruses, as determined using the tissue culture infective dose (TCID₅₀) method, were 1.9×10^7 mL⁻¹ for adenovirus type 5, 4.6×10^7 mL⁻¹ for RSV type A2, 3.2×10^7 mL⁻¹ for rhinovirus type 13, and 2×10^7 mL⁻¹ for influenza A type H1N1. The virus preparations were further diluted in RPMI media, and 100 μ L aliquots were added to the purified macrophages in 96-well plates at a multiplicity of infection (MOI) of 3. We chose an MOI of 3 because preliminary studies in our laboratory showed that, unlike MOIs of 1 and 10, it produced a consistent effect when comparing all viruses under uniform conditions.

As negative control, 100 μ L of tissue culture media without virus (sham medium) was added to the macrophage culture. As positive control, macrophages were incubated with 5 ng of lipopolysaccharide (LPS). The macrophage cultures were then incubated at 37°C, and at 3 and 6 h, 50 μ L of supernatant was aspirated and assayed for IL-6 and TNF α by quantitative multiplex enzyme-linked immunosorbent assay (Biosource, Camarillo, CA). All experiments of macrophage stimulation from each donor were performed in triplicate. To calculate the concentrations of newly produced cytokines in the supernatant from virus-stimulated macrophage cultures, the concentrations of IL-6 and TNF α contained in the original virus inoculum were subtracted. The concentrations of cytokines released by macrophages were analyzed by the nonparametric Mann-Whitney Rank Sum test. *P* values of 0.05 were considered significant.

Figure 1 (top two panels) shows the results of IL-6 released by macrophages. Sham stimulation was associated with minimal amounts of IL-6 at 3 and 6 h of incubation, and the difference between polymorphic (G/C or C/C) and normal (G/G) donors was not significant. LPS stimulation, as a positive control, induced significant amounts of IL-6, which were higher in polymorphic donors; this effect was significant at 6 h. Overall, mean concentrations of IL-6 stimulated by all viruses were higher in those with polymorphic genotype compared with normal donors; this effect was significant for RSV at both 3 and 6 h.

An interesting pattern was observed with RSV stimulation. As shown in Table 1, analysis of the individual donor experiments performed in triplicate showed that 6 of 7 (86%) donors with normal IL-6 genotype produced little or no IL-6 (at least 2 of 3 wells showing no IL-6 production) at 3 h; these donors were termed "poor responders." In contrast, only 2 of 7 (29%) donors with polymorphic IL-6 genotype were poor responders. The differences between polymorphic and normal genotypes were statistically significant (*P* = 0.002, *Z*-test). The observation of poor responders persisted at 6 h in 5 of 7 (71%) donors with normal genotype compared with 2 of 7 (29%) with polymorphic genotype (data not shown).

Three of 7 donors with normal IL-6 genotype were also polymorphic for TNF α ; 2 of these donors were good responders, suggesting that TNF α polymorphism can influence the production of IL-6 in those with normal IL-6 genotype who would otherwise be expected to be poor responders. However, because of the small number of donors in these subgroups, only limited conclusions can be drawn. No other

TABLE 1. INTERLEUKIN-6 CONCENTRATIONS (PG/ML) IN THE SUPERNATANT FLUIDS OF MACROPHAGE CULTURES STIMULATED BY RESPIRATORY SYNCYTIAL VIRUS FOR 3 H

	Number of triplicate wells with detectable IL-6	Mean $\pm$ SD (pg/mL)
Normal donors		
V20	3/3	1,104 $\pm$ 400
V33	0/3	0
V36	0/3	0
V37	0/3	0
V38	1/3	8 $\pm$ 14
V39	0/3	0
V41	0/3	0
IL-6 ⁻¹⁷⁴ donors		
V8	1/3	18 $\pm$ 31
V10	3/3	773 $\pm$ 613
V21	3/3	2,683 $\pm$ 385
V22	3/3	1,083 $\pm$ 486
V27	2/3	232 $\pm$ 357
V34	3/3	1,368 $\pm$ 668
V40	0/3	0

RSV multiplicity of infection = 3; 1×10^5 macrophages/well; final volume = 200 μ L. The mean IL-6 concentrations were calculated after subtracting the baseline concentration of IL-6 contained in the RSV inoculum (when the calculated values were equal to or less than the RSV inoculum concentration, a value of zero was assigned).

IL-6, interleukin-6; SD, standard deviation; RSV, respiratory syncytial virus.

combinations of genotypes were analyzed. For all other virus stimulations, the phenomenon of poor responders was not observed.

Figure 1 (bottom two panels) shows the results of TNF α released by macrophages. Sham stimulation was associated with minimal amounts of TNF α at 3 and 6 h of incubation; however, the difference between polymorphic (G/A or A/A) and normal (G/G) individuals was significant at 6 h. LPS stimulation, as positive control, induced significant amounts of TNF α ; however, the difference between polymorphic and normal donors was not significant. The mean concentrations of TNF α stimulated by all viruses were higher in those with a polymorphic genotype. This effect was significant for RSV at both 3 and 6 h, and for adenovirus and influenza at 6 h. The effect of IL-6 polymorphic genotypes on TNF production was not analyzed because of the small number of donors in these subgroups.

Our *in vitro* model of macrophage-virus interaction allowed direct observation of the effect of cytokine gene polymorphisms on cytokine production induced by respiratory viruses without the modulating influences of other cellular elements that are found at the tissue infection site. The present study shows for the first time that IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ polymorphic genotypes are associated with enhanced production of cytokines when macrophages are exposed to common respiratory viruses. This effect was most significant for RSV in IL-6⁻¹⁷⁴ donors and for adenovirus, influenza virus, and RSV in TNF α ⁻³⁰⁸ donors.

The ability of common respiratory viruses, such as RSV, adenovirus, rhinovirus, and influenza virus, to induce the synthesis of IL-6 and TNF α by APCs such as monocytes, macrophages, and dendritic cells has been reported (Midulla

and others 1989; Lehmann and others 1996; Thiele and others 2006; Laza-Stanca and others 2006), but the effect of cytokine genotypes of donors have not been described. The enhanced *in vitro* production of IL-6 and TNF α by respiratory viruses in polymorphic donors shown in the present study can explain the previously observed association of IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ genotypes with susceptibility to respiratory viral infection and its complications. For example, we have shown that both IL-6⁻¹⁷⁴ and TNF α ⁻³⁰⁸ polymorphic genotypes are associated with higher susceptibility to recurrent AOM (Patel and others 2006). Alper and others (2009) have also shown that TNF α ⁻³⁰⁸ genotype increases the risk for AOM following rhinovirus URI. Gentile and others (2003) have shown that, in children hospitalized with RSV bronchiolitis, IL-6⁻¹⁷⁴ genotype was associated with prolonged hospitalization and lower O₂ saturation. Puthothu and others (2009) have shown that several TNF polymorphic haplotypes increase the risk for a more severe course of bronchiolitis due to RSV infection. However, none of these studies evaluated the direct interaction between virus and the host cell with polymorphic genotypes.

There is evidence from published reports about the inhibitory potential of RSV. For example, we have previously reported the ability of RSV to inhibit IL-1 activity and adhesion molecules expression (Roberts and others 1986; Salkind and others 1991). These results are consistent with our present observation of suppressed production of IL-6 following RSV stimulation in donors with the normal genotype. This phenomenon of poor response was not observed with other viruses or with TNF α production. It is possible that RSV suppresses the specific signal pathways for IL-6 transcription and translation, but the IL-6⁻¹⁷⁴ polymorphic genotype is able to overcome this inhibition at the transcription level. This phenomenon requires further study.

Cytokine gene polymorphisms may also influence the immunogenicity and reactogenicity orchestrated by tissue macrophages interacting with respiratory viral vaccine antigens. For example, Poland and others (2008) have shown that specific IL-6 polymorphic genotypes were associated with better H1- and H3-specific hemagglutination inhibitory antibody response to inactivated seasonal influenza A vaccine (A/H1N1 New Caledonia/20/99, A/H3N2 Panama/2007/99). On the other hand, TNF polymorphisms were not associated with immune response or vaccine adverse events. However, no *in vitro* studies have been reported to date on the study of interactions between viral vaccine antigens and host gene polymorphisms, and the subsequent impact on the function of APCs such as macrophages.

Our study design has certain limitations. For example, the requirement for exposure to live virus and intracellular replication was not determined. Also, the role of soluble mediators, including immunoregulatory cytokines, in the tissue culture-grown viruses was not investigated. However, our *in vitro* model mimics the *in vivo* model of infection in that the APCs are expected to be exposed to the viruses in the respiratory tract that are produced by the host respiratory epithelial cells along with the various soluble mediators, including cytokines. Further studies are also needed to examine the role of polymorphic genotypes of IL-1, another acute-phase cytokine, as well as interactions between various cytokine genotypes and the inflammatory cascade.

In summary, our study shows that the production of IL-6 and TNF α by macrophages exposed to common respiratory viruses is significantly regulated by the high-cytokine-producing genotypes. This, in turn, may have an impact on the susceptibility to, severity of, and recovery from respiratory virus infections.

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Author Disclosure Statement

The authors do not have a commercial or other association that might pose a conflict of interest.

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Address correspondence to:

Dr. Janak A. Patel

Division of Pediatric Infectious Disease and Immunology

Department of Pediatrics

University of Texas Medical Branch

Galveston, TX 77555-0371

E-mail: jpatel@utmb.edu

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