SHORT COMMUNICATION

Commonly invasive serotypes of *Streptococcus pneumoniae* trigger a reduced innate immune response compared with serotypes rarely responsible for invasive infection

Thomas S.T. Burgess[1], Aaron F. Hirschfeld[1], Gregory J. Tyrrell[2], Julie A. Bettinger[1] & Stuart E. Turvey[1]

[1]Department of Pediatrics, Division of Infectious and Immunological Diseases, BC Children's Hospital and Child & Family Research Institute, University of British Columbia, Vancouver, BC, Canada; and [2]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada

Correspondence: Stuart E. Turvey, Department of Pediatrics, Division of Infectious and Immunological Diseases, BC Children's Hospital and Child & Family Research Institute, Rm 371, 950 West 28 Avenue, Vancouver, BC, Canada, V5Z 4H4. Tel.: +1 604 875 2345 ext 5094; fax: +1 604 875 2226; e-mail: sturvey@cw.bc.ca

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Abstract

Although there are more than 90 serotypes of *Streptococcus pneumoniae* (or pneumococcus), it is not understood why a small number of serotypes account for most invasive infections. To investigate the human innate immune response triggered by different pneumococcal serotypes, monocyte-derived macrophages were exposed to a group of commonly and rarely invasive pneumococcal clinical isolates and tumor necrosis factor (TNF)-α production was measured. Commonly invasive pneumococcal serotypes triggered significantly less TNF-α production than serotypes rarely responsible for invasive infection ($P < 0.004$). These data indicate that one factor influencing the invasive potential of a pneumococcal serotype is the magnitude of innate immune-mediated TNF-α production triggered by exposure to the organism and suggest that the integrated host response generated against commonly invasive pneumococcal serotypes may be less effective than the response directed against rarely invasive serotypes.

Streptococcus pneumoniae, also known as pneumococcus, is a pathogen of global importance causing invasive infections such as sepsis, meningitis, and pneumonia. More than 90 immunologically distinct serotypes of pneumococcus have been described, each varying in the structure of their polysaccharide capsule. However, the prevalence with which the serotypes are recovered from patients with invasive disease varies considerably, presumably because some serotypes have a much greater propensity to cause invasive disease than others. International pneumococcal surveillance has demonstrated that 70–88% of invasive pneumococcal disease in young children was caused by serotypes now included in the seven-valent protein-conjugated vaccine formulation (4, 6B, 9V, 14, 18C, 19F, and 23F) (Henriques *et al.*, 2000; Hausdorff *et al.*, 2005; Bettinger *et al.*, 2007). This finding is consistent with the hypothesis that, regardless of ethnic and socioeconomic differences, 'commonly invasive' serotypes as a group have a high level of intrinsic virulence relative to other serotypes.

Emerging evidence suggests that innate immune mechanisms and particularly Toll-like receptor (TLR) signalling are critical for defense against pneumococcus. Indeed, both humans with inherited immunodeficiencies affecting innate immunity (Picard *et al.*, 2003) and mice with engineered defects affecting innate immune recognition (Echchannaoui *et al.*, 2002; Malley *et al.*, 2003; Albiger *et al.*, 2005) show enhanced susceptibility to invasive pneumococcal infection. Given that innate immunity provides the sophisticated first line of defense against infection and empowers the subsequent adaptive immune response, it follows that the ability of the innate immune system to recognize different pneumococcal serotypes may influence which serotypes are most commonly responsible for invasive infections. In this study, it was sought to investigate how commonly invasive serotypes of pneumococcus interact with human monocyte-derived macrophages to trigger an innate immune response, compared with pneumococcal serotypes rarely responsible for invasive infection. Specifically, macrophage production of the inflammatory cytokine tumor necrosis factor (TNF)-α

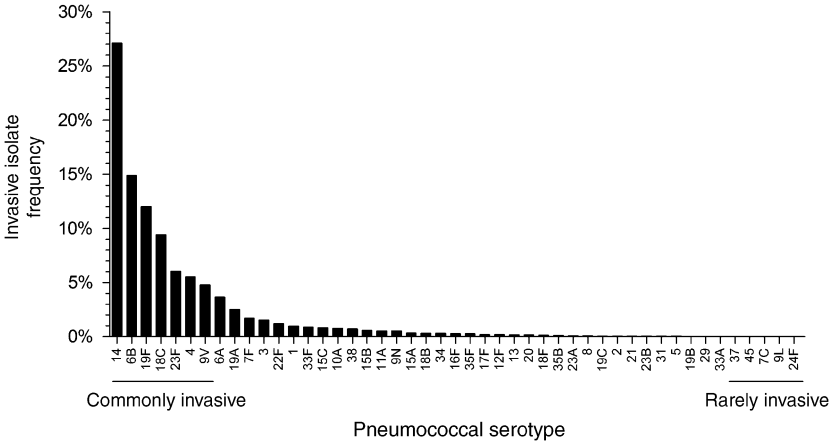

Fig. 1. Distribution of invasive pneumococcal serotypes in Canadian children, 1991–2004. From January 1991 to September 2004, the Immunization Monitoring Program, Active (IMPACT) has collected data on a total of 3528 cases of invasive pneumococcal infection in children 0–17 years of age across Canada. The distribution of invasive pneumococcal serotypes among IMPACT cases is shown. Serotypes marked as 'commonly invasive' and 'rarely invasive' were used in this study.

was quantified following exposure to clinical isolates of pneumococcus. Monocyte-derived macrophages were studied as these are key innate immune cells encountered by bacteria during the evolution of an invasive infection, and the production of the proinflammatory cytokine TNF-α was quantified as TNF-α production is known to influence the outcome following pneumococcal infection (Takashima *et al.*, 1997; Wellmer *et al.*, 2001).

Based on Canadian epidemiological data (Fig. 1), a library of 'commonly invasive' serotypes (14, 6B, 19F, 18C, 23F, 4, 9V) was acquired that collectively account for 80% of invasive pneumococcal infections in Canadian children, and 'rarely invasive' serotypes (37, 45, 7C, 9L, 24F) that as a group only caused 0.1% of the cases of invasive disease (Fig. 1). These pneumococcal serotypes were all isolated from the blood of patients experiencing an invasive pneumococcal infection. Formal growth curves were established for each *S. pneumoniae* serotype. For macrophage stimulation, pneumococcal serotypes were grown in brain-heart infusion (BHI) medium to the mid-log phase ($A_{600\,nm} = 0.3$), harvested by centrifugation, killed in 70% ethanol (v/v) for 1 h on ice, washed again, and resuspended in lipopolysaccharide-free phosphate-buffered saline. Inhibition of bacterial replication was necessary to control for host responses that could be attributed to differential growth rates of live bacteria *in vitro* rather than growth-independent differences due to the serotype alone. Ethanol killing was selected to preserve pneumolysin, an ethanol-stable but heat-labile TLR4 ligand (Malley *et al.*, 2003).

Human monocyte-derived macrophages were prepared by differentiating THP-1 cells (ATCC #TIB-202) toward a macrophage phenotype in the presence of phorbol myristate acetate (PMA) (Tsuchiya *et al.*, 1982). THP-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and 1 mM sodium pyruvate (Gibco). Forty-eight hours before bacterial stimulation, the THP-1 cells were differentiated with PMA (0.1 μM) and after 24 h the cells were washed and allowed to rest in fresh medium. THP-1 (2×10^5) cells were incubated (in triplicate) in a flat-bottom 96-well plate with ethanol-killed pneumococci at varying multiplicities-of-infection (MOIs). After 24 h, the supernatant was harvested and the TNF-α concentration was quantified by an enzyme-linked immunosorbent assay (ELISA) (eBioscience). Stimulation with each serotype was performed at least twice in triplicate. An MOI of 200 bacteria to 1 THP-1 cell (i.e. MOI = 200 : 1) was demonstrated to optimally trigger secretion of TNF-α while avoiding cytotoxicity as demonstrated by the lactate dehydrogenase (LDH) cytotoxicity assay (data not shown).

When the ability of different pneumococcal serotypes to trigger innate immune-mediated TNF-α secretion by monocyte-derived macrophages was compared, commonly invasive pneumococcal serotypes induced significantly less proinflammatory cytokine production than serotypes rarely responsible for invasive infection (commonly invasive serotypes: mean TNF-α concentration = 369 pg mL^{-1}, SEM = 38 pg mL^{-1}, $n = 42$; rarely invasive serotypes: mean TNF-α concentration = 712 pg mL^{-1}, SEM = 50 pg mL^{-1}, $n = 30$. $P < 0.004$ by Student's *t*-test. Fig. 2).

These data suggest that a difference in innate immune-mediated cytokine production is likely to be one factor influencing the capacity of pneumococcal serotypes to cause invasive infection. TNF-α secretion as a consequence of innate immune activation is known to orchestrate a variety of early and late protective host responses against infection, including triggering the initial acute phase response, empowering the adaptive immune response through maturation of dendritic cells, and activating endothelial cells to enhance effector leukocyte recruitment (Locksley *et al.*, 2001). Hence, these data suggest that the integrated host response generated against commonly invasive pneumococcal serotypes may be less effective than the response directed against rarely invasive serotypes. The observation that commonly invasive serotypes of pneumococcus induce less

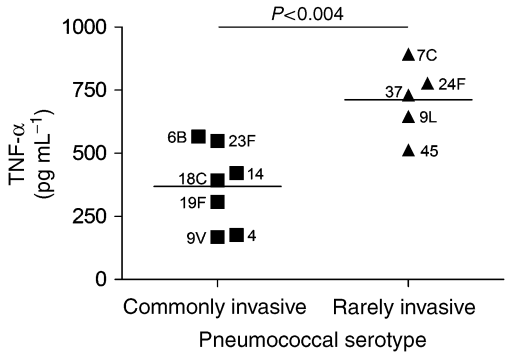

Fig. 2. Commonly invasive pneumococcal serotypes induce significantly less pro-inflammatory TNF-α production than serotypes rarely responsible for invasive infection. Human monocytic cells (THP-1) were stimulated with a variety of pneumococcal serotypes at an MOI of 200 : 1. Clinical pneumococcal isolates were classified as either commonly or rarely invasive based on previous epidemiological studies. After 24 h of exposure to the pneumococci, culture supernatants were harvested and TNF-α production was quantified by ELISA. All bacterial stimulations were performed in triplicate and repeated twice. Values represent mean TNF-α production from all experiments. Groups were compared by Student's *t*-test and $P < 0.004$.

robust TNF-α production is particularly intriguing, given that a wide spectrum of microorganisms have acquired elegant mechanisms to interfere with host responses mediated by TNF-α (Rahman & McFadden, 2006). In this study, the authors elected to measure TNF-α secretion following pneumococcal exposure, as production of TNF-α is known to influence the outcome in experimental models of pneumococcal infection (Takashima *et al.*, 1997; Wellmer *et al.*, 2001). Importantly, it has been demonstrated previously that production of a variety of cytokines (specifically IL-1β, IL-6, and IL-10) is well correlated with TNF-α secretion following innate immune activation of human mononuclear cells (Hirschfeld *et al.*, 2007).

In this study, pneumococcal serotypes were classified as either 'commonly invasive' or 'rarely invasive' using basic epidemiological surveillance data (Fig. 1). However, alternative pneumococcal classification schemes have been proposed. For example, a published alternative is to divide pneumococcal serotypes into 3 major groups on the basis of their ability to cause invasive disease in humans (Sandgren *et al.*, 2004). The first group includes serotypes most commonly found in invasive disease and rarely found in carriers – such as serotypes 1, 4, and 7F – that have a high invasive disease potential. The second group includes serotypes commonly found both in invasive disease and in carriers – such as serotypes 14 and 6B – that have an intermediate invasive disease potential. The third group includes serotypes primarily found in carriers – such as serotype 19F – that have a low invasive disease potential. Another possible classification scheme is by clonal properties. The particular clonal type, in addition to capsular serotype, may be an important determinant of potential to cause disease. In one study, serotype 14 included clones that were only found among carriers, as well as clones causing only invasive infection, suggesting that different clones have different potential for disease (Sandgren *et al.*, 2004).

Ultimately, the ability of different pneumococcal serotypes to colonize and cause invasive disease is likely to depend on multiple host and pathogen factors. It is demonstrated that the capacity to trigger an innate immune response is one factor that influences the potential of a specific pneumococcal serotype to cause invasive infection (Fig. 2). Nevertheless, other factors certainly influence the evolution of invasive pneumococcal infection. For example, generation of specific antibody against the polysaccharide capsule is important in determining the frequency of invasive infection as some of the most commonly invasive pneumococcal serotypes, such as 6B, 19F, and 23F, are slow to induce high titer antibody responses (Soininen *et al.*, 2001). However, mechanisms in addition to antibody production must be at play, as serotype 14 is both commonly invasive and highly immunogenic in terms of antibody induction (Soininen *et al.*, 2001).

A potential weakness of this study is that the authors quantified the innate immune response generated by only one cell type – human monocyte-derived macrophages – following exposure to *S. pneumoniae*. In the process of causing invasive infection, pneumococci must interact with a variety of host cells and the most immunologically active of these are respiratory epithelial cells, monocytes, macrophages, and dendritic cells. Therefore, it would be valuable to expand the authors' studies to include analysis of innate immune responses generated by human respiratory epithelial cells and human dendritic cells following exposure to different pneumococcal serotypes.

Despite vaccination strategies, pneumococcus remains the leading cause of invasive bacterial infections in children and the elderly. Moreover, rising rates of antibiotic nonsusceptibility around the world make this infection increasingly more difficult to treat with currently available antimicrobial agents. New treatment and prevention strategies are likely to arise through an improved understanding of the basic immunological mechanisms involved in invasive pneumococcal disease, and the present data indicate that one factor influencing the invasive potential of a pneumococcal serotype is the magnitude of innate immune-mediated TNF-α production triggered by exposure to the organism.

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