INFECTION AND INFLAMMATION: POTENTIAL TRIGGERS OF SUDDEN INFANT DEATHS

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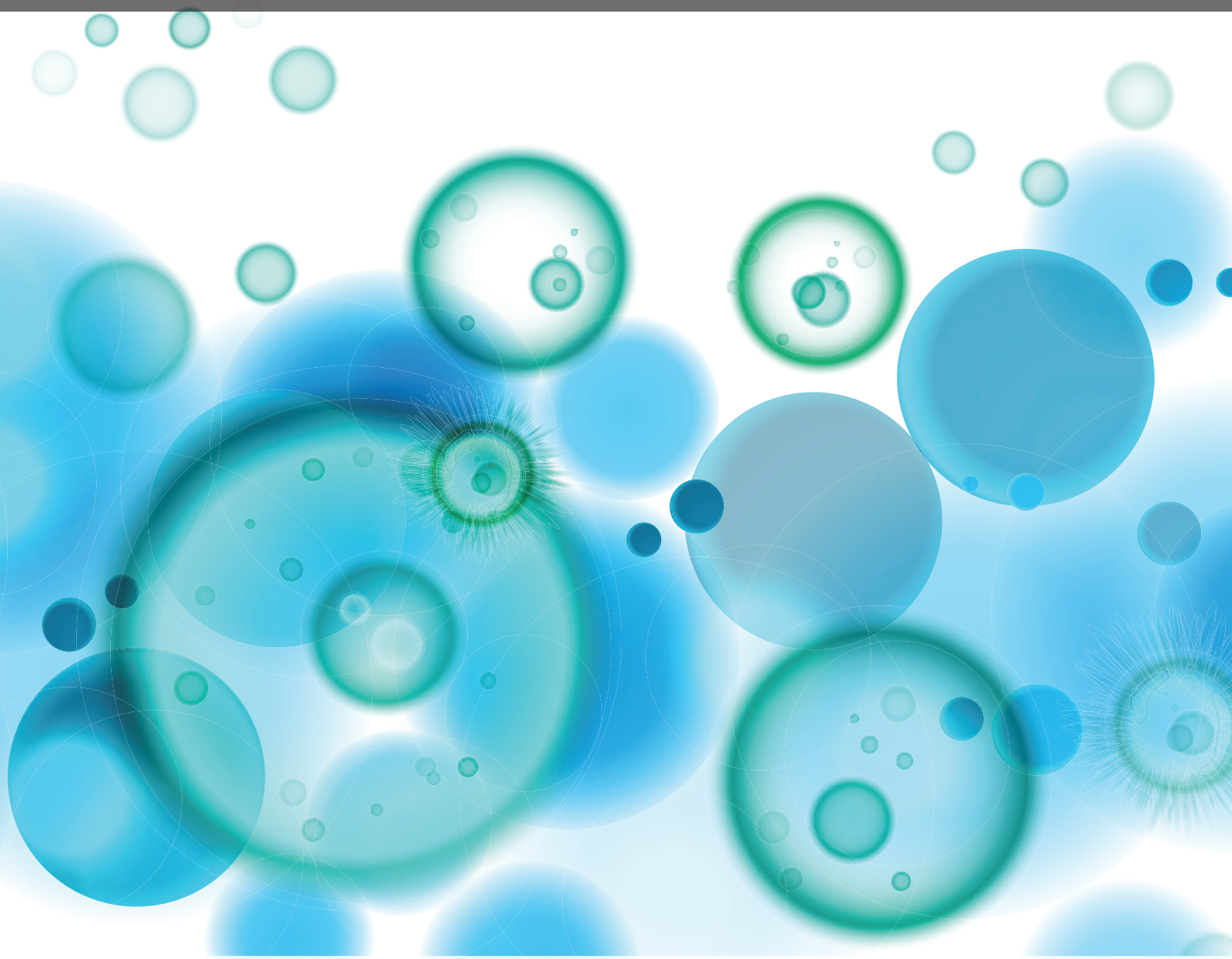

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INFECTION AND INFLAMMATION: POTENTIAL TRIGGERS OF SUDDEN INFANT DEATHS

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EDITORIAL
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Editorial: Infection and Inflammation: Potential Triggers of Sudden Infant Deaths

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Keywords: sudden infant death, infection inflammation, cytokines, genetics, developmental stage

The Editorial on the Research Topic

Infection and Inflammation: Potential Triggers of Sudden Infant Deaths

Among infants in industrialized countries, sudden infant death syndrome (SIDS) is still the major cause of death between 1 month and 1 year of age. SIDS is a diagnosis of exclusion, a subset of sudden unexpected deaths in infancy (SUDI), those deaths that remain unexplained following a thorough postmortem, assessment of the medical history, and investigation of the scene of death. The risk factors for SUDI/SIDS identified in epidemiological studies parallel risk factors associated with susceptibility to infection in children, and various studies have identified microorganisms or their products in significant proportions of these deaths (Blackwell et al.). Even in a country, such as Hungary, with a historically low incidence of SUDI/SIDS, there is evidence that infection and inflammation contribute to these deaths (Töro et al.). This collection provides insights into a variety of approaches for the investigation of how genetic, environmental, and developmental risk factors could dysregulate or potentiate the effects of inflammatory responses to "mild" infections that are often reported prior to death of the infant.

Exploration of the role of infectious agents in these deaths has been limited by two factors. The evidence does not fit Koch's postulates: no single organism has been isolated from all cases of SUDI/SIDS. A variety of organisms, both viruses and bacteria with various virulence factors, has been identified in different studies (Blackwell et al.; Bettelheim and Goldwater; Goldwater). In addition, there is no animal model that accurately reflects all the genetic, developmental, and environmental risk factors associated with SUDI/SIDS. This problem and examples of the models developed are reviewed by Blood-Siegfried. The common thread in the investigation of infection does not appear to be a specific infectious agent but the infant's inflammatory response to the infection. Therefore, it is important to develop models to assess risk factors that can influence levels of inflammatory mediators, which can affect the major physiological mechanisms proposed to explain SUDI/SIDS – anaphylaxis, poor arousal, hypoxia and apnea, shock, cardiac arrhythmias, hyperthermia, and hypoglycemia.

The differences in the incidence of SIDS among ethnic groups and the excess of male infants indicated that genetic factors might be involved in triggering these deaths. Potential genetic contributions to these deaths and the case for whole exome sequencing are assessed here by Morris. Genetic variations in the immune/inflammatory responses in relation to SUDI are reviewed by Ferrante and Opdal. The potential contribution of two cytokines and polymorphisms in their genes, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), is explored by Moscovis et al. and Moscovis et al. in relation to these responses to infectious agents and interactions with environmental risk factors such as cigarette smoke. The differences in cytokine responses of male and female fetuses identified by Burns et al. indicate that females might be better able to control inflammatory responses *in utero*.

Two important risk factors for SUDI/SIDS are low birth weight and premature birth. These were explored in relation to two environmental factors that could affect fetal development during

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pregnancy, exposure to cigarette smoke, and evidence of inflammation. Evidence of inflammation was inversely correlated to gestational age. Exposure to cigarette smoke was also significantly associated with low birth weight and age at delivery. The effects of both evidence of inflammation and exposure to cigarette smoke were more pronounced for male infants (Pringle et al.).

In the absence of a widely accepted model for these infant deaths, their investigation requires the cooperation of a variety of disciplines to attempt to identify why these children have died suddenly. Current evidence indicates that inflammatory responses are dysregulated by combinations of infectious agents and cigarette smoke. Developmental stage of the infant and hormone levels might also affect these responses (Blackwell et al.).

The approaches and techniques developed to examine SUDI/SIDS need to be applied to stillbirths, which have been suggested to be part of the spectrum of infant deaths associated with SIDS (1). While the risk factors are similar, investigations of stillbirths need to take into consideration the responses to infectious agents of both the mother and the infant (Blackwell).

New techniques emerging for studies of infection and inflammation will complement the standard studies carried in routine forensic examinations: assessment of microbiome for both viral and bacterial pathogens; whole genome sequencing of DNA from infants; and screening for a range of inflammatory mediators. These techniques are not usually available for diagnosis of infant deaths. Progress in this area will require cooperation between forensic medicine and pathology services and research groups with the expertise to complement investigations carried out by standard protocols.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Exploring the risk factors for sudden infant deaths and their role in inflammatory responses to infection

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The risk factors for sudden infant death syndrome (SIDS) parallel those associated with susceptibility to or severity of infectious diseases. There is no evidence that a single infectious agent is associated with SIDS; the common thread appears to be induction of inflammatory responses to infections. In this review, interactions between genetic and environmental risk factors for SIDS are assessed in relation to the hypothesis that many infant deaths result from dysregulation of inflammatory responses to "minor" infections. Risk factors are assessed in relation to three important stages of infection: (1) bacterial colonization (frequency or density); (2) induction of temperature-dependent toxins; (3) induction or control of inflammatory responses. In this article, we review the interactions among risk factors for SIDS for their effects on induction or control of inflammatory responses. The risk factors studied are genetic factors (sex, cytokine gene polymorphisms among ethnic groups at high or low risk of SIDS); developmental stage (changes in cortisol and testosterone levels associated with 2- to 4-month age range); environmental factors (virus infection, exposure to cigarette smoke). These interactions help to explain differences in the incidences of SIDS observed between ethnic groups prior to public health campaigns to reduce these infant deaths.

Keywords: sudden infant death syndrome, inflammation, infection, cigarette smoke, ethnicity

INTRODUCTION

Sudden infant death syndrome (SIDS) is still the major cause of death between 1 month to 1 year of age among infants in industrialized countries. SIDS is a diagnosis of exclusion. The original definition was "... the sudden death of any infant or young child, which is unexpected by history, and in which a thorough post mortem examination fails to demonstrate an adequate cause of death" (1).The definition was revised in 1989 to "the sudden death of an infant under one year of age, which remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history" (2) Comparison of epidemiological data from different countries found that infants of some ethnic groups had an increased risk of SIDS (**Table 1**).

The pathogenesis of SIDS has been examined by many disciplines. These have made significant contributions to the study of infant deaths and put forward hypotheses; however, many cannot explain the risk factors or the positive effects of the public health campaigns to reduce the risk (10). The idea that inflammation might be involved in these infant deaths is not new. In an article published in 1956, 126 non-traumatic sudden ("unexplained") infant deaths were investigated; 106 (84%) revealed microscopic inflammatory changes in 1 or more sites of the respiratory tract, and there was histologic evidence of inflammatory disease in other organs in many cases (11). **Table 2** summarizes some of the evidence for inflammatory responses in sudden infant deaths.

RISK FACTORS FOR SIDS AND SUSCEPTIBILITY TO INFECTION

Epidemiological studies found significant associations between SIDS and a variety of genetic, developmental, and environmental factors (**Table 3**). When these factors are compared with those associated with increased susceptibility to bacterial infections, there are remarkable parallels. Each of these factors can affect one or more of the important stages in susceptibility to or severity of infections: frequency or density of bacterial colonization of mucosal surfaces; induction of temperature-dependent toxins; induction or control of inflammatory responses to infection. Each of these is described in detail below.

Infectious agents or their products have been identified during autopsies of SIDS infants and more recently in sudden unexpected death of infancy (SUDI), which is defined as the sudden and unexpected death of an infant under 1 year of age; SUDI includes explained deaths and those that, after investigation of the death scene and meticulous post-mortem examination, remain unexplained (42). For many years, microbiological findings were dismissed as contamination, overgrowth, or normal flora. More recent studies have questioned these assertions and have provided evidence to support the likelihood that post-mortem bacteriology is a true reflection of infection in these infant deaths (42–44). The common bacterial toxin hypothesis (45–47) considered a number of the risk factors in a mathematical model. It has been supported by identification of potentially toxigenic bacteria or their toxins in

Table 1 | Variation in the incidence of SIDS among ethnic groups within countries before the "reduce the risks" campaigns.

Country	Ethnic group	SIDS/1,000 live births	Reference
Australia	Aboriginal	6.1	(3)
	Non-aboriginal	1.7	
United Kingdom	European	1.7	(4)
	Bangladeshi	0.3	
United States	Total population	2	(5–7)
	Oriental	0.3	
	African-American	5.0	
	Native-American	5.9	
	Alaskan-Natives	6.3	
New Zealand	Maori	7.4	(8, 9)
	Non-Maori	3.6	

Table 2 | Inflammatory or immune responses identified in SIDS infants.

Organ system	Response	Reference
Respiratory tract	Peribronchial inflammatory infiltrates	(12, 13)
	Increase in IgM producing cells in trachea	(14)
	Mast cell degranulation	(15) (Walls, this issue)
Digestive tract	Increased IgA producing cells in duodenum	(14)
	Increased salivary IgA	(16)
Nervous system	Interferon alpha in neurons of the medulla of the brain	(17)
	Increased levels of IL-6 in spinal fluid	(18)
	Lymphocyte infiltration	(19)
Blood	Decreased IgG response to bacterial toxins	(20, 21)
	Increased IgM response to core endotoxin	(21)
	Increased levels of mast cell tryptase	(15)
	Increased levels of mannose binding lectin	(22)
	Cross-linked fibrin degradation products	(23)

Table 3 | Risk factors for SIDS that parallel risk factors for susceptibility of infants to infection.

Risks	Reference
Genetic	
Ethnicity	(3–7, 9, 24)
Male gender	(25–27)
Developmental	
Night time deaths	(28, 29)
Peak age range 2–4 months	(25)
Environmental	
Prone sleeping	(25, 30)
Cigarette smoke exposure	(25, 28)
Overheating	(31)
Mild respiratory infections	(25, 31, 32)
Lack of breast feeding	(33)
Poor socio-economic conditions	(25, 34)
No or late immunization	(35, 36)
Air pollution	(37)
Used cot mattress	(38, 39)
Day care	(40)
Older siblings	(41)

Table 4 | Toxigenic bacteria and their toxins implicated in sudden death in infancy.

	Toxin	Reference
Staphylococcus aureus	Enterotoxins, TSST[a]	(48–50)
Bordetella pertussis	Pertussis toxin, endotoxin[a]	(51–53)
Haemophilus influenzae	Endotoxin[a]	(54)
Clostridium perfringens	Enterotoxin A[a]	(55, 56)
Clostridium botulinum	Botulism toxin	(57–59)
Streptococcus pyogenes	Pyrogenic toxins A and B[a]	(54)
Escherichia coli	Enterotoxins, verotoxins	(60–63)
	Curlin[a]	(64)
Helicobacter pylori	Endotoxin, vacuolating[a] toxin, urease	(65)
Pneumocystis	?	(66)

[a] *Superantigenic activity.*
?, toxin/antigen unknown.

SIDS and sudden unexpected deaths in infancy (SUDI) (**Table 4**). Many of the bacterial toxins or components of the bacteria implicated can act as super antigens, eliciting powerful cytokine storm responses such as those seen in toxic shock syndrome or bacterial sepsis.

The infection hypothesis does not fit Koch's postulates. That is, there is no single organism implicated and there is no widely accepted animal model that reflects the genetic, developmental, and environmental risk factors identified in epidemiological studies (see Blood-Siegfried, this issue). In addition, a variety of viruses have been identified in SIDS infants (67–69). Sterile site infections have been identified in SUDI, and a variety of toxigenic bacteria or their toxins have been reported in these infant deaths (**Table 4**). Our hypothesis is that there is not a particular organism or toxin, the factor in these deaths is the dysregulation of the inflammatory responses elicited by what appear to be mild or asymptomatic infections.

The objective of this review was to assess how the risk factors identified in epidemiological studies of SIDS affect susceptibility to infection and/or alter inflammatory responses to infections. It addresses the interactions between these identified risk factors and the three key stages of infection: (1) increased frequency or density of bacterial colonization; (2) induction of temperature-dependent toxins; (3) induction or control of inflammatory responses (**Table 5**).

Table 5 | How do risk factors for SIDS affect susceptibility to infection.

Effects on frequency or density of colonization by bacteria by
Developmental stage/expression of receptors
Prone position
Virus infection
Cigarette smoke
Day care
Older siblings
Effects on temperature-dependent bacterial toxin production by
Overheating
Prone position
Virus infection
Effects on induction or control of inflammatory responses by
Cigarette smoke
Virus infection
Sex
Genetic background
Developmental stage (low maternal antibodies, cortisol levels, testosterone surge)

BACTERIAL COLONIZATION

Factors affecting colonization have been elucidated in previous studies. Virus infections, which often precede SIDS or SUDI, can enhance bacterial binding through induction of host receptors for bacteria or induction of new receptors (70, 71). The prone position can lead to pooling of respiratory secretions and increased numbers and varieties of bacteria, particularly in the presence of virus infection (72). Active smoking can predispose individuals to virus infections, and smokers are colonized more heavily and more frequently with potential pathogens (73). In addition to enhancing susceptibility to virus infections, material in cigarette smoke can passively coat epithelial cells and enhance "stickiness" for potential pathogens (74). Exposure of infants to new infectious agents can be enhanced by day care with other children or older siblings attending nursery or school outside the home environment.

TEMPERATURE-DEPENDENT TOXINS

The pyrogenic toxins of *Staphylococcus aureus* have been identified in over half of SIDS infants from five different countries (50). *S. aureus* is the most common isolate from the nasopharynx of healthy infants, and 64% of these have the capacity to produce these toxins (75). The toxins are induced only between 37 and 40°C, and the temperature of the nasopharynx is usually below this range (76). In the prone position, the temperature in the nasopharynx of children is increased and 15% had temperatures $\geq$37°C. While the toxigenic organisms are present in most infants, induction of the toxin is likely to be dependent on risk factors such as overheating, prone position, or virus infection with associated blocked nasal passages that result in reduced cooling effects of the passage of air.

INFLAMMATION AND SIDS

The common thread in these deaths is not a single organism or toxin; it is dysregulation of the innate inflammatory responses in the non-immune infant or young child who encounters a new potential pathogen. There is recent evidence that a balanced inflammatory response during infancy is important for survival. In a study of low birth weight infants, cytokine responses were assessed *in vitro* by stimulation of whole blood cultures with lipopolysaccharide (LPS), phytohemagglutinin (PHA), or purified protein derivative (PPD). Infants whose cytokine responses to LPS or PHA were very low or very high were at increased risk of death assessed by survival of the cohort tested. The interpretation of these findings was that a balanced response to new pathogens was more likely to result in survival (77). A variety of bacteria implicated in SIDS/SUDI possess structural antigens or exotoxins that can act as superantigens (e.g., LPS) that can activate large populations of inflammatory cells (**Table 3**).

Inflammation can be assessed from clinical evidence, histological findings, or molecular markers. Clinical evidence of inflammation has been identified in some of these infant deaths. Infants were very hot when found and remained hot for several hours after death (78). Histological signs of inflammation are considered the gold standard by the clinical pathologist and some of these are summarized in **Table 2**. It has been suggested that molecular markers of inflammation need to be measured; however, these are relatively expensive and are not usually included in standard autopsy protocols. To date, there have been few studies of inflammatory markers in sudden deaths (18, 79, 80). Because these components are not regularly examined during autopsy, cooperation with a research group with an interest in inflammation would help to obtain evidence for these markers.

INFLAMMATION IN RELATION TO PROPOSED MECHANISMS OF DEATH IN SIDS

Cytokines produced in the inflammatory responses to infectious agents could have an impact on most of the mechanisms of death proposed: anaphylaxis; poor arousal; hypoxia and apnea; shock; cardiac arrythmias; hyperthermia; hypoglycemia (81). Anaphylaxis was the first mechanism proposed in an animal model in which guinea pigs were sensitized to cow's milk (82, 83). Studies of SIDS infants have not found evidence to support the hypothesis that IgE-mediated anaphylaxis is involved in these infant deaths. There is evidence, however, for degranulation of mast cells in some infants (15) (Walls, this issue). In addition, in an *in vitro* study of first degree relatives of SIDS infants or infants who had suffered an acute life threatening episode (ALTE), there was evidence of increased mast cell hyper-releasability and degranulation (84). This could be mediated by some of the pyrogenic staphylococcal toxins through non-antibody activation of mast cells. The toxins, which are super antigens, can bind directly to the Vβ antigens on mast cells and trigger degranulation independent of antibody and complement. A mild virus infection might enhance colonization by *S. aureus* and increased temperature of the oropharynx due to blocked nasal passages. This could result in temperatures that are permissive for induction of the staphylococcal toxins. Increased production of interferon-γ (IFN-γ) and/or other mediators could enhance expression of Vβ antigens which are receptors for the toxins. Cross-linking of the Vβ antigens by the toxins could lead to non-IgE-mediated degranulation of mast cells (**Figure 1**). Evidence of mast cell tryptase and other products of mast cells have been published (15) (Walls, this volume).

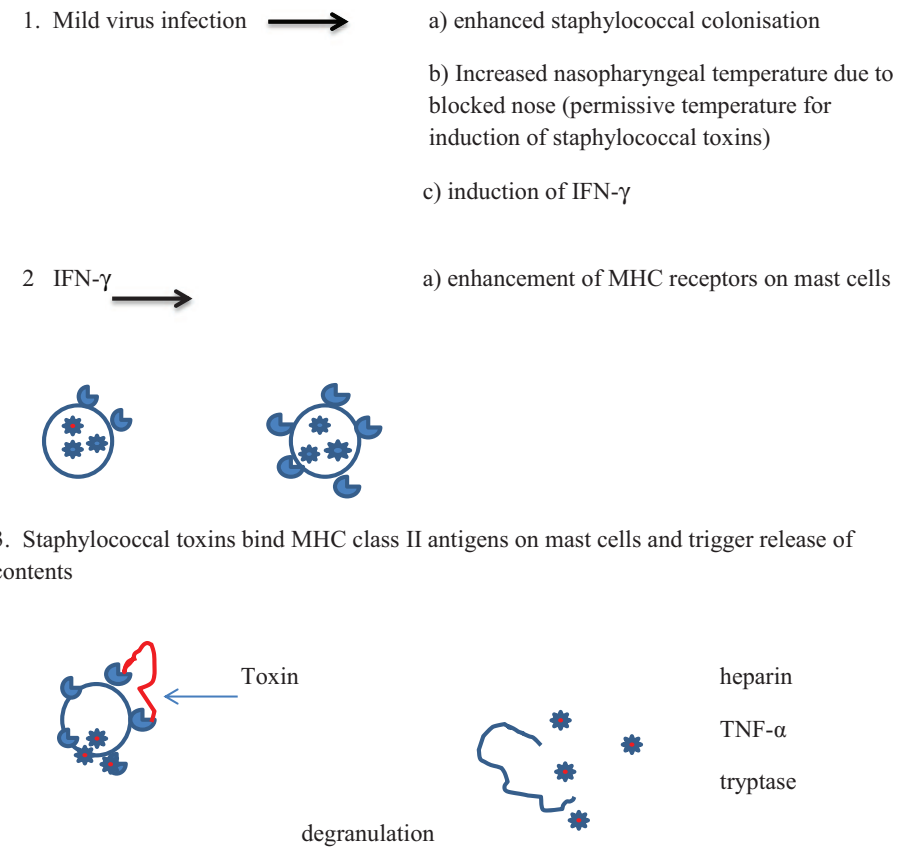

FIGURE 1 | Proposed scheme for interactions between virus infections and pyrogenic staphylococcal toxins in non-antibody-mediated induction of mast cell degranulation (see text).

IN VITRO STUDIES OF INFLAMMATION IN HUMAN MATERIAL

The inflammatory responses can be affected by genetic, developmental, and environmental factors. *In vitro* studies need to consider these potential confounding factors. Taking blood from young infants is not practical for most studies, so cell lines or adult leukocytes have been used to assess the effects of risk factors for SIDS on inflammation. In addition to controlling for confounding factors, it is important to use biologically relevant concentrations of substances to be assessed in the model system (85).

While bacteria and their toxins have been implicated in SIDS, there is epidemiological and experimental evidence for virus infections acting as a cofactor. Many parents reported their child had a mild cold or sniffle prior to death. The presence of a risk factor such as prone sleeping, head covered, or parental smoking combined with infection was associated with a greater risk of SIDS than the individual risk factor alone (32).

In animal models, the lethal doses of both staphylococcal exotoxins (86) and endotoxin (87–89) were significantly reduced if the animals had mild/asymptomatic virus infection. Virus infections induce IFN-γ, which can significantly enhance production of pro-inflammatory cytokines (Moscovis et al., this volume). IL-6 has been identified in the CSF of SIDS infants (18). We demonstrated a dose-dependent enhancement by IFN-γ of IL-6 elicited by LPS in a human cell line and human peripheral blood monocytic cells (PBMC) (85). In addition, in a model system, IFN-γ has been demonstrated to reduce significantly the anti-inflammatory IL-10 responses elicited from human PBMC exposed to endotoxin (Moscovis et al., this volume).

In contrast, components of cigarette smoke, such as nicotine (or its liver metabolite cotinine), can suppress endotoxin-induced IFN-γ, IL-1β, TNF-α, and IL-8. There are, however, limitations in using single purified reagents for *in vitro* studies as the responses elicited might not reflect accurately the complex interactions that occur *in vivo*. Nicotine is only one of approximately 4,000 chemicals in cigarette smoke. A water soluble cigarette smoke extract (CSE) enhanced IL-8 responses but reduced other pro-inflammatory cytokines (85). *In vitro* studies found cells from smokers produced significantly lower anti-inflammatory IL-10 responses than cells of non-smokers (90). This could impair the ability to control pro-inflammatory responses to infection.

DEVELOPMENTAL STAGE AND SIDS

The mathematical model used for the common bacterial toxin hypothesis predicted the 2- to 4-month age range as that during which the peak of SIDS occurred. This prediction fits the

epidemiological data. There are a number of factors that could contribute to susceptibility to infection during his period: (1) loss of maternal antibody; (2) development of circadian rhythms and the associated changes in night time cortisol; (3) the testosterone surge in males between 1 and 5 months.

ANTIBODY LEVELS AND THE EFFECTS OF IMMUNIZATION

An important factor is the loss of maternal antibodies, which make the infants more reliant on their innate inflammatory responses to cope with new infections. This can include exposure to new infections brought home by older siblings or attendance at day care, both of which have been identified as risk factors for SIDS in epidemiological studies. Immunization has been demonstrated to be a protective factor in relation to SIDS (35). Most infants start their immunizations during the period of peak vulnerability, but they will not be fully immunized to common childhood illnesses implicated in some epidemiological studies (e.g., whooping cough) by 4 months of age. The protective effect of immunization was noted in early studies (35), and a shift in the age range of SIDS in infants in both Scotland and England was observed following changes in the immunization of infants in UK. That is, immunization began at 2 months instead of 3 months (91). It has also been demonstrated that some cross protection against staphylococcal exotoxins was elicited in animals by tetanus toxoid (91).

DEVELOPMENT OF CIRCADIAN RHYTHM AND HORMONAL CHANGES

During the first months of life, significant physiological changes occur which could affect control of the inflammatory responses. Circadian rhythm develops between 4 and 16 weeks in Caucasian infants in Britain, but this does not occur until 12–20 weeks in Asian infants (92). The switch to circadian rhythm is measured by the night time drop in core body temperature. This physiological switch is accompanied by a dramatic drop in night time, but not daytime, cortisol levels (**Figure 2**).

The levels of night time cortisol after the switch were not capable of reducing pro-inflammatory responses in a model system examining inflammatory responses of peripheral blood monocytes to staphylococcal toxins (93). Daytime levels and night time levels prior to the switch were able to reduce these responses. If these *in vitro* studies reflect the activity of cortisol *in vivo* in the infant, this 2- to 4-month period could be a window of vulnerability to new infections, particularly at night. The circadian switch occurs later among Asian infants (12–20 weeks), which allows time for natural exposure to infection or immunization to boost specific immunity. Prior to the public health campaigns to reduce the risks of SIDS, there was a lower incidence of SIDS among Asian infants in UK compared with infants of European origin (4).

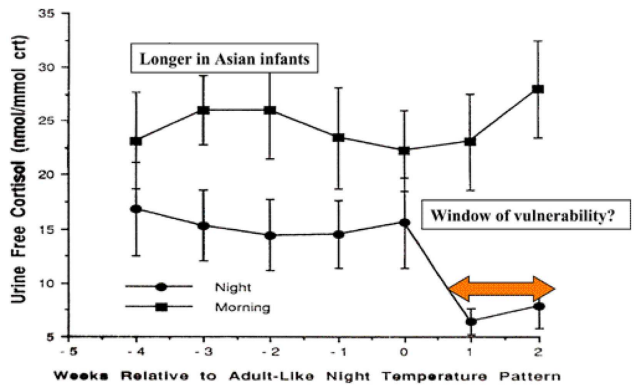

FIGURE 2 | Day time and night time cortisol levels of infants in relation to development of circadian rhythm and potential for control of inflammatory responses to infection [adapted from Ref. (92)].

THE EFFECT OF TESTOSTERONE

The male excess in SIDS noted in epidemiological studies and assessed in mathematical models (26) might reflect hormonal changes that occur during the period in which SIDS is most prevalent. There is a rise in testosterone production associated with the period during which SIDS is most prevalent. Between 1 and 5 months, testosterone levels range from 0.03 to 6.14 nMol L^{-1} for males and 0.03 to 0.17 nMol L^{-1} for females. In males, these levels decrease to 0.07–0.24 at 6–11 months (94). In a previous study (95), the ranges of testosterone in the adult females (<0.4–3.1 nMol L^{-1}) tested were within the range for males in the 1- to 5-month age range (94). In the model system examined, there was a positive correlation between testosterone levels and pro-inflammatory responses to LPS when the cells were pretreated with IFN-γ or IFN-γ and CSE. No correlations were observed for the higher levels (6.3–21 nMol L^{-1}) found in adult males (95).

These findings indicate that in addition to low levels of night time cortisol present in infants over the age range of greatest risk of SIDS (93), dysregulation of the inflammatory responses to apparently "mild" infections might be amplified by the increase in testosterone in male infants at this time. As this does not occur in female infants, this could be an additional factor contributing to the higher proportion of males among SIDS infants. *In vitro* studies using the PBMC model might provide additional insights into the interactions between cortisol and testosterone levels noted in infants during this critical age range.

INTERACTIONS WITH GENETIC FACTORS

Among the ethnic groups at increased risk of SIDS (e.g., indigenous populations in Australia and North America and Black Americans), there is a higher proportion of genotypes associated with strong pro-inflammatory responses (96–98). While these disparities have been ascribed primarily to socio-economic disadvantage, there is emerging evidence that genetic background and interactions between environmental factors such as cigarette smoke might contribute to susceptibility and severity of infections (85, 90). Of particular importance is the effect of cigarette smoke on the genotype of IL-10 (G-1082A) associated with lower levels of the anti-inflammatory response. The AA genotype is predominant among the groups at higher risk of SIDS, and in our *in vitro* studies, cells from smokers with this genotype had the lowest responses (90). Among the ethnic groups at increased risk of SIDS, there is also a higher incidence of maternal smoking. South Asians have genetic profiles similar to those for the higher risk groups; however, smoking is much less prevalent among south Asian women in UK.

Our experimental studies indicate that higher IFN-γ responses might elicit higher pro-inflammatory responses to bacterial toxins (Moscovis et al., this volume). Indigenous Australians tested had the highest proportion of individuals with the TT genotype of *IFNG* T + 874A (96), which is associated with high IFN-γ responses observed *in vitro* (99).

CONCLUSION

There is a wealth of knowledge about the risk factors for sudden death in infancy; it is important, however, to attempt to explain how these risk factors could result in death. There is a growing body of evidence that infection and inflammatory responses might trigger the events leading to sudden death in infancy. A recent review on infectious causes of SIDS concluded no specific organism was involved in SIDS (100). The evidence of Anderson et al. (77) indicates that a balanced inflammatory response is important for dealing with new infections. Our hypothesis is that the common thread in these deaths is dysregulation of the inflammatory responses to apparently mild infections. The risk factors identified in epidemiological studies can have significant effects on inflammatory responses that could affect the different physiological mechanisms proposed to explain these infant deaths (81). Many of the genetic, developmental, and environmental factors identified could affect this balance resulting in enhanced pro-inflammatory cytokine levels, which can affect glucose levels, heart rate, apnea, arousal, anaphylaxis, and shock. The components of the inflammatory responses involved might differ in individual children; however, it is the dysregulation of the responses that lead to the death of the child. We need to investigate further the interactions between genetic, developmental, and environmental risk factors on inflammatory responses to attempt to identify infants at increased risk and to attempt to introduce measures to prevent induction of these lethal responses.

AUTHOR CONTRIBUTIONS

Each of the authors made substantial contributions to the conception, design, analyses, and interpretations of the work. They assisted in preparing the article, critically assessed the final version, and agree to be accountable for the accuracy and integrity of the work.

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Evidence for infection and inflammation in infant deaths in a country with historically low incidences of sudden infant death syndrome

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Total infant mortality in Hungary has been higher than other European countries; however, the reported incidence of sudden infant death syndrome (SIDS) has been lower. The low incidence of SIDS in Hungary has been supported by evidence obtained from the high rate of scene of death investigation and medico-legal autopsy mandatory since the 1950s. In this study, we compared the incidence of explained and unexplained infant deaths in Hungary for three periods: 1979–1989 when the incidence of SIDS was high in western Europe; 1990–1999 when the incidence of infant deaths was falling following introduction of the public health campaigns to reduce the risk factors associated with SIDS; and 2000–2012 to determine if introduction of *Haemophilus influenzae* type b or pneumococcal vaccines or introduction of an earlier immunization schedule during this period had an effect on SIDS. Explained infant deaths fell consistently during this period; however, SIDS rose during the second period when the incidence of SIDS was falling in other European countries. Evidence for infection and/or inflammation was observed for the majority of SIDS during each period. The results are discussed in relation to campaigns to reduce infant mortality in Hungary and the introduction of new vaccines and an earlier immunization schedule in 2006.

Keywords: sudden infant death syndrome, infant mortality, immunization, infection, inflammation

Introduction

In 2004, we reported that infant mortality in Hungary was higher than many European countries (1) (**Figure 1**) and it has remained high according to the OECD figures for 2014 (2); however, the reported incidence of sudden infant death syndrome (SIDS) has been lower (0.15–0.3/1000 live births) than those of other countries (1, 3, 4). The historically low incidence of SIDS in Hungary has been supported by evidence obtained from the high rate of scene of death investigation and medico-legal autopsy.

It was reported that prior to 1980, the incidence of SIDS was lower in East Germany and this was attributed to a lower prevalence of prone sleeping compared with figures for West Germany (5).

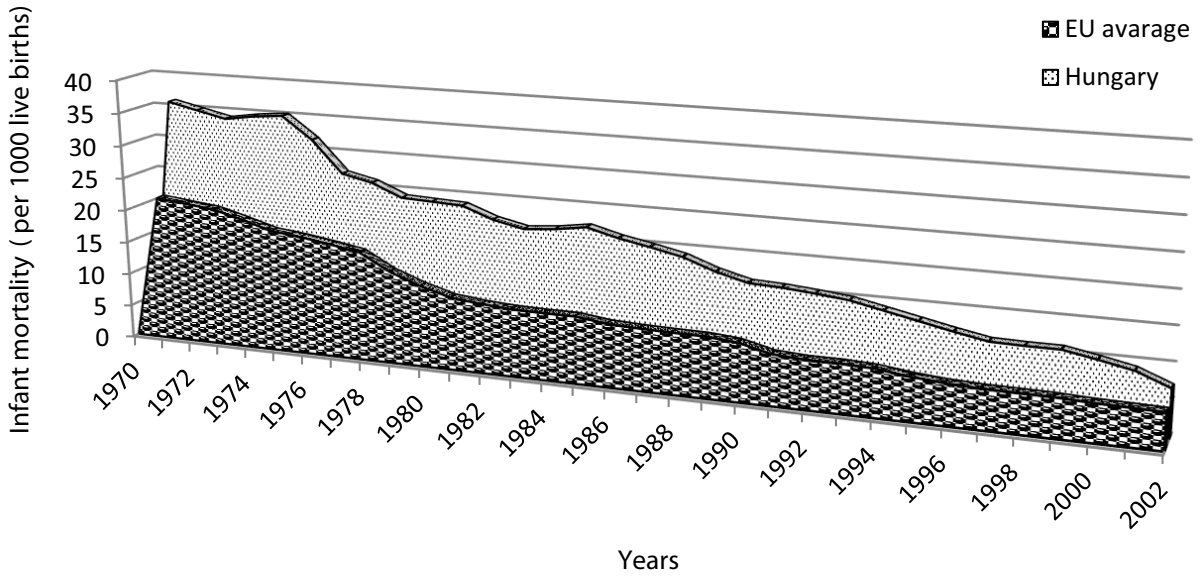

FIGURE 1 | Comparison of infant mortality (per 1000 live births) for Hungary and the European Union (data from the WHO Regional Office for Europe).

SIDS has many risk factors that parallel susceptibility to infectious diseases, and there is increasing evidence that infection and inflammatory responses play a role in the events leading to these deaths (Blackwell et al., this issue). During 1983–1989, the incidence of SIDS in Norway and Sweden appeared to parallel the incidence of whooping cough. Mortality rates followed significantly the monthly prevalence of *Bordetella pertussis* in Sweden where immunization for pertussis had been discontinued. In Norway where pertussis immunization was continued, this correlation was less obvious (6).

In relation to these observations, it has been suggested that the historic emphasis on infant immunization in Hungary might have contributed to the lower incidence of sudden deaths triggered by infectious agents. Immunization began in the late eighteenth century with variolization. Immunization with vaccinia replaced this practice, and the first mandatory vaccine for children in Hungary was for small pox in 1876. Tetanus toxoid was offered from 1940, but this was not mandatory.

A national immunization plan (NIP) has been in effect in Hungary since the mid-1950s and mandatory since its introduction; costs are covered by the national budget and exemptions are allowed only for medical reasons. The NIP began with universal BCG vaccine for infants at the maternity hospitals prior to discharge. This was followed by diphtheria-pertussis (whole cell)-tetanus (DPT), subsequently replaced in 2006 by an acellular pertussis vaccine. Immunizations were at 3, 4, and 5 months, with boosters at 3 and 6 years until 2006. Since then, the primary doses are given at 2, 3, and 4 months, and the boosters are given at 18 months and 6 years.

Haemophilus influenzae type b (Hib) prophylaxis began in 1999 (3, 4, and 5 months) in conjunction with DPT immunization. In 2006, the combined acellular DPT, inactivated polio and Hib vaccine was introduced. Pneumococcal conjugate vaccine (7-valent) was introduced in 2008 at 2, 4, and 18 months. It was free but not mandatory; however, the uptake was 83% for all eligible infants. The 7-valent vaccine was replaced by the 13-valent *n* in 2010.

The current study compared explained deaths due to infection and those due to SIDS in three periods: 1979–1989 when the incidence of SIDS was higher in western Europe; 1990–1999 when the incidence of infant deaths was falling following introduction of the public health campaigns to reduce the risk factors identified for SIDS (e.g., prevention of prone sleeping, overheating, and exposure to cigarette smoke); and 2000–2012 to determine if introduction of Hib or pneumococcal vaccines had an effect on SIDS. Available autopsy records were assessed for evidence of infection and inflammation among infant deaths in these three periods. As initiation of infant immunization in the United Kingdom was associated with a shift in the age ranges of SIDS infants (7, 8), data on age of SIDS infants in central Hungary were analyzed to determine if there was a similar decrease in the proportion of SIDS infants in the age range of 3–4 months following the change to the earlier immunization schedule.

Materials and Methods

In Hungary since the 1950s, all sudden infant deaths are referred for medico-legal investigations and autopsy. Data on SIDS and deaths due to infection were collected from the Hungarian National Statistics Office Database for three periods: period 1, 1979–1989; period 2, 1990–1999; and period 3, 2000–2012. The ninth and tenth revisions of the international classification of diseases (ICD) were used for the determination of cause of death (**Table 1**). SIDS cases were classified as 798.0 (ICD version ninth) or R95 (ICD version tenth). ICD codes are derived from the autopsy records and death certificates. The ninth version covered the years between 1979 and 1995. The tenth version was introduced in 1996.

TABLE 1 | Selected ICD codes versions 9 and 10 reflected to the infections in different organs.

ICD code version		
9	**10**	**Cause of death**
001–139	A00–B99	Certain infections and parasitic diseases
320–357	G00–G08	Diseases of the nervous system
360–389	H65–H70	Diseases of the ear and mastoid process
460–519	J01–J69	Diseases of the respiratory system
520–579	K35–K75	Diseases of the digestive system
580–629	N10–N39	Diseases of the genitourinary system
682–709	L00–L99	Diseases of the skin and subcutaneous tissue
710–739	M00–M99	Diseases of the musculoskeletal system
760–779	P23–P77	Certain conditions originating in the perinatal period
798.0	R95	Sudden infant death syndrome

Sudden infant death syndrome cases were diagnosed by the currently accepted definition (9). If severe inflammation or signs of septicemia were noted, these cases were not classified as SIDS. Infection and inflammation in different organ systems were analyzed and assigned to ICD codes (**Table 1**).

Results

In the three time periods, there were 47412 infant deaths: period 1, 26124; period 2, 13433; and period 3, 7855. In each period, the majority of deaths were explained and a small number were classified as SIDS: period 1, 251 (0.9%); period 2, 331 (2.4%); and period 3, 271 (3.4%). For each period, there was an excess of male deaths among the SIDS infants: period 1, 155 (62%) male, 96 (38%) female); period 2, 193 (58%) male, 138 (42%) female; and period 3, 154 (57%) male, 117 (43%) female (**Table 2**). Most SIDS cases (70%) occurred between 2 and 6 months of age. The rate of mild infections among SIDS victim under 6 months old was 70%, while the proportion was only slightly lower (60%) among older SIDS infants.

Respiratory infections (J01–J69) were the most common cause of death in all three periods; however, there was a steady decline, particularly after the introduction of the Hib vaccine in 1999 and the pneumococcal vaccine in 2006 (**Figure 1**). For each of the three periods, there was a higher proportion of males than females; however, the difference was not significant.

In contrast to the steady decline in deaths due to respiratory infections, there was an increase in the number and proportion of SIDS deaths in period 2 (2.4%) compared to those of period 1 (0.9%). This was followed by a decrease in numbers of SIDS in period 3, but an increase in the proportion of total deaths (3.4%) (**Figures 2A,B**). The increase in SIDS cases in the second period was the opposite of the decrease in SIDS cases in other countries in Europe. While the average SIDS per 1000 live births was 0.235, males were more likely to die of SIDS (0.268) than females (0.2), (**Figure 3**).

Among the explained deaths, the proportion in which infection was identified decreased significantly from 13.6% in period 1 to 8.4% in period 3 ($X^2 = 168.9$, df = 2, $p = 0$). As noted in the methods, if severe inflammation or signs of septicemia were noted, these cases were not classified as SIDS. For SIDS, the proportion of infants with evidence of mild infection did not vary significantly: 66% in period 1; 65% in period 2, and 58% in period 3 ($X^2 = 4.57$, df = 2, $p = 0.1$) (**Table 3**).

A decrease in SIDS noted in the United Kingdom following initiation of infant immunization at 2 months rather than 3 months, particularly in the age range of 3–4 months. Between 1994 and 2012, there were 114 SIDS in the Budapest-Central Hungary area; 70 between 1994 and 2005 and 44 between 2006 and 2012. The immunization of infants at 2 months of age began in 2006. As with figures reported for the UK, the greatest decrease in SIDS was noted for children aged 3 months 14/70 (20%) prior to change in the immunization schedule to 2/42 (4.5%) in the second period (Fishers exact probability test, two tailed 0.026). While the numbers were small, they reflected the pattern noted in other studies.

Discussion

In Hungary in the first and second periods examined, the main focus was to reduce infant mortality from known causes; it was much higher than in western European countries (1). The main strategy was to reduce perinatal mortality. Prevention of infections through immunization was an important part of this strategy and deaths from infection have declined (**Table 1**; **Figure 2**). These efforts were reflected in reduction in deaths due to meningitis, septicemia, and pneumonia. In contrast to other European countries, there was an increase in the proportion of SIDS in period 2 (1990–1999), followed by a decrease in period 3. For periods 1 and 2, evidence of mild infection was noted for over 60% of unexplained infant deaths investigated compared to 13.6 and 10.9% for infectious deaths in the comparable periods. In studies in the 1990s, *Streptococcus pneumoniae* was isolated from a significantly higher proportion of SIDS infants (11%) compared to healthy infants matched for age, sex, and locality (10). While rare, pneumococcal infections have been implicated in sudden infant deaths (11). It was noted in our data that there was a sharp decline in SIDS following introduction of the pneumococcal vaccine in 2008 (**Figure 3**) and it has remained below the numbers prior to 2008. Although the numbers were small, the decrease in the numbers of deaths at 3 months also decreased dramatically following initiation of infant immunization at 2 months in 2006 and, while the numbers are small they have not returned to levels noted prior to 2006.

There was no year when the SIDS rate per 1000 live births was more than 0.4/1000. The increase in SIDS in Hungary was not expected and requires investigation. There are several factors to be considered.

In the 1990s, there was a campaign based on studies in other countries to reduce the risk factors for SIDS. There have been no case-control studies in Hungary similar to those in other countries on which the "reduce the risks" campaigns were based. In Hungary, the prone sleeping position was not widely accepted as a real risk factor for SIDS. The risk factors for SIDS most widely accepted as significant were infection, lack of immunization, crowded living conditions associated with lower social-economic status, and exposure to cigarette smoke.

Reduction of exposure of infants to cigarette smoke was strongly emphasized in the campaign; however, compared to statistics available for Britain, the proportion of women in

TABLE 2 | Number of deaths during the three periods assessed by ICD classification and gender.

Selected ICD codes	Number of deaths (%)						
	1979–1989		1990–1999		2000–2012		1979–2012
	Male	Female	Male	Female	Male	Female	Male + Female
A00–B99	135 (0.9)	109 (1.0)	112 (1.5)	67 (1.2)	40 (0.9)	27 (0.8)	490 (1.0)
G00–G08	176 (1.2)	153 (1.4)	100 (1.3)	76 (1.3)	32 (0.7)	29 (0.8)	566 (1.2)
H65–H70	184 (1.2)	130 (1.2)	39 (0.5)	37 (0.6)	12 (0.3)	15 (0.4)	417 (0.9)
J01–J69	1144 (7.7)	797 (7.1)	423 (5.6)	262 (4.5)	111 (2.6)	82 (2.3)	2819 (5.9)
K35–K75	81 (0.5)	57 (0.5)	23 (0.3)	16 (0.3)	6 (0.1)	4 (0.1)	187 (0.4)
N10–N39	10 (0.1)	9 (0.1)	11 (0.1)	5 (0.1)	1 (0.0)	1 (0.0)	37 (0.1)
L00–L99	6 (0.0[a])	4 (0.0[a])	0 (0.0)	1 (0.0[a])	0 (0.0)	0 (0.0)	11 (0.0[a])
M00–M99	0 (0.0)	1 (0.0[a])	0 (0.0)	0 (0.0)	1 (0.0[a])	0 (0.0)	2 (0.0[a])
P23–P77	488 (3.3)	361 (3.2)	235 (3.1)	184 (3.2)	235 (5.4)	205 (5.8)	1708 (3.6)
R95	155 (1.0)	96 (0.9)	193 (2.5)	138 (2.4)	154 (3.6)	117 (3.3)	853 (1.8)
TOT	14949	11175	7617	5816	4316	3539	47412

[a]*Percentage <0.1%.*

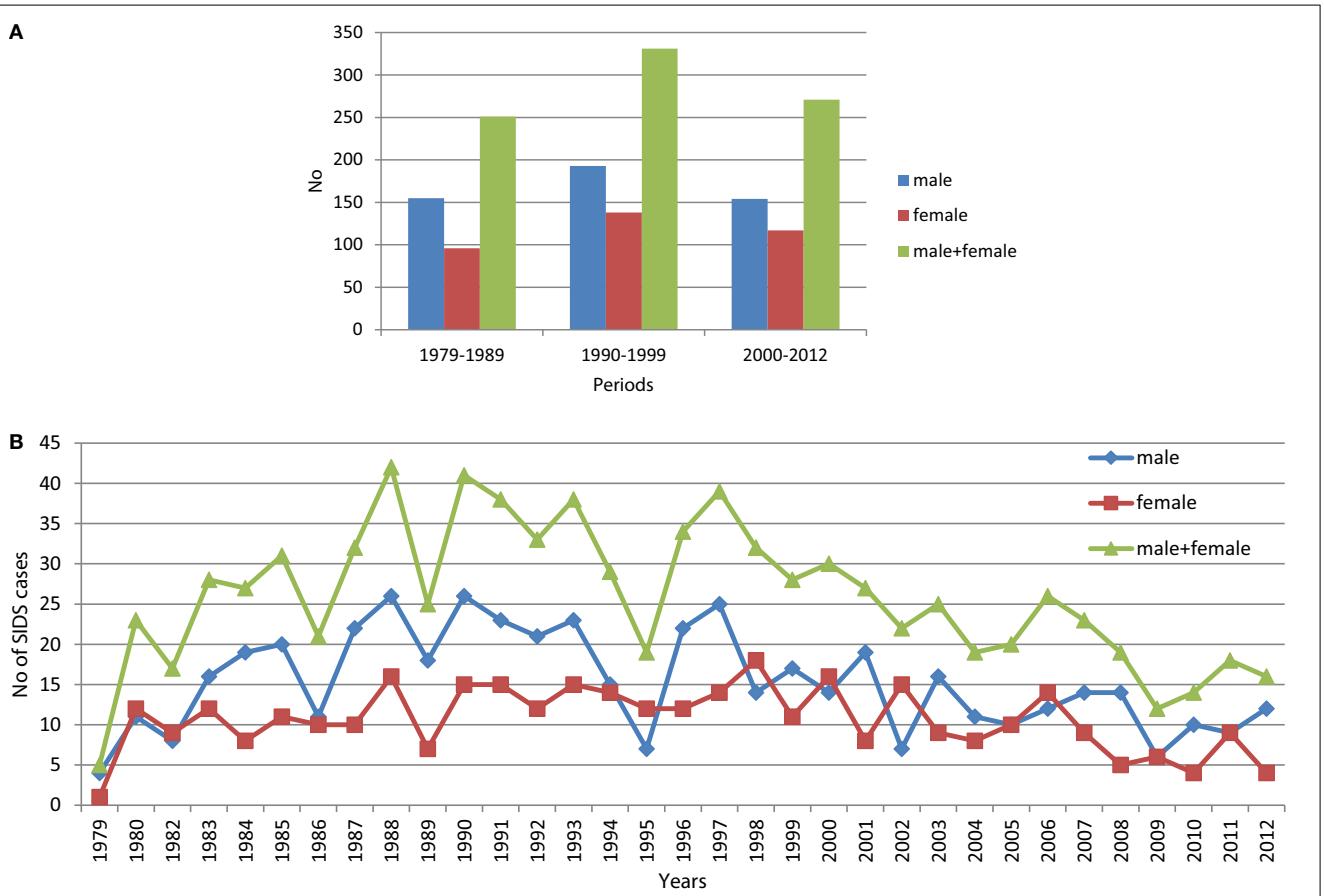

FIGURE 2 | Percentage distributions deaths due to respiratory diseases (J01–J69). (A) Number of SIDS cases in the three periods investigated. **(B)** Number of SIDS cases per years between 1979 and 2012.

Hungary who smoke has not changed from the mid-1970s. Smoking among women decreased in the UK from 41% in 1974 to 17% in 2013 (12). For Hungary, there has not been a similar decline: 22% of women smoked in 1975 and 24% smoked in 2012 (13).

During and after the campaign, pathologists and forensic pathologists began to accept more widely that SIDS is a credible diagnosis. Documentation and the scene of death investigation became much more rigorous, not only to exclude infanticide or accidents but also to obtain data on symptoms of illness prior to death, prenatal and postnatal diseases, and disorders in development. Investigation focused not only on the pathomorphological changes but also evaluated the history of

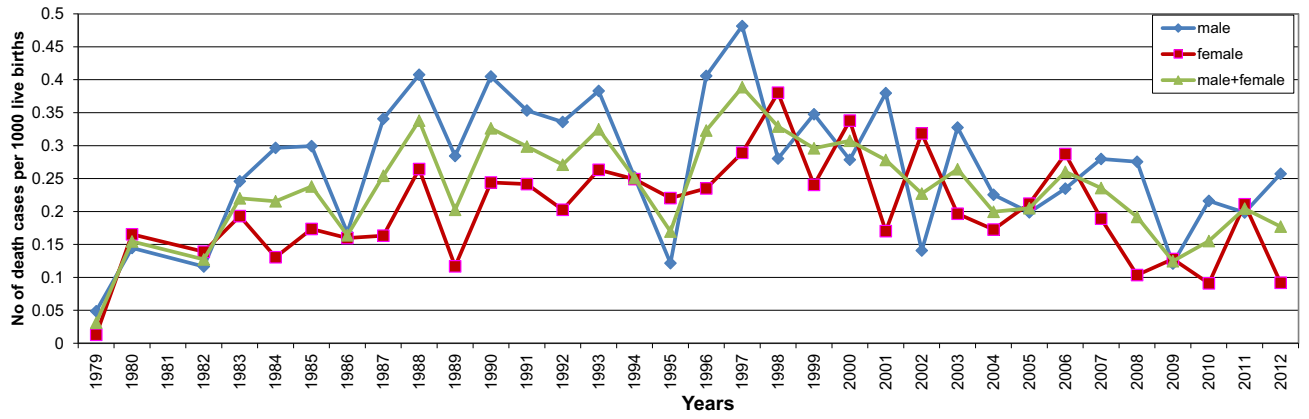

FIGURE 3 | Distribution of SIDS cases per 1000 live births 1979–2012.

TABLE 3 | Analyses of evidence of infection in infant deaths in Hungary.

	1979–1989 No (%)	1990–1999 No (%)	2000–2012 No (%)
Explained*	25873	13202	7584
Infection	3521 (13.6)	1451(10.9)	637 (8.4)
Unexplained (SIDS)	251	331	271
Mild infection	166 (66)	215 (65)	157 (58)

**Microbiologically confirmed and accepted.*

pregnancy and delivery. Postmortem microbiology was also introduced.

Although there was no standard autopsy protocol, the autopsy and histology in every case of sudden infant death is compulsory. Due to the increase in emphasis on accurate diagnosis, the possibility of change in diagnosis or bias cannot be ruled out; however, the age range affected, the excess of male deaths, and the pathomorphology (congested lungs, number and distribution of petechiae, liquid heart blood) were similar to those observed in Hungary prior to the intervention and in other countries. While the proportion of infection-related explained deaths declined during the three periods studied, the proportion of SIDS infants with evidence of minor infection/inflammation remained constant at approximately 60%.

There is evidence of infection in many of these infants. Bacteria or nuclear bodies in some indicating viral infections can be demonstrated microscopically. Bacterial and viral cultures have not been given much weight in assessment of SIDS because of the concern that the organisms are contaminants or overgrowth of normal flora and not related to the cause of death. More recent studies have identified bacteria in normally sterile sites in investigations of sudden death in infancy (14–16). Most of these organisms do not continue to grow when a body is stored at 4°C. In addition, staphylococcal toxins produced only at temperatures between 37 and 40°C have been identified in significant proportions of SIDS infants from different countries including Hungary (17) and toxigenic enteric organisms have also been identified in these infants (Bettelheim and Goldwater, this issue). Recent studies have indicated that virus infections might enhance inflammatory responses to mild bacterial infections (Moscovis et al., this issue). Autopsies need to include assessment of the molecular signs of inflammation such as cytokines that can affect the various physiological mechanisms proposed to explain these deaths (Blackwell et al., this issue).

In recent years, the autopsy rates in many countries have decreased, including Hungary. This is unfortunate as there is a growing persuasive argument for including new investigative techniques that could contribute to explaining the cause of these deaths: genetic investigations (Morris, this issue); and detailed microbiological investigations, both conventional diagnostic procedures and more extensive molecular screening techniques (Goldwater, this issue; Bettelheim and Goldwater, this issue).

Conclusion

The current study compared explained deaths due to infection and those due to SIDS in three periods: 1979–1989 when the incidence of SIDS was higher in western Europe; 1990–1999 when the incidence of infant deaths was falling following introduction of the public health campaigns to reduce the risk factors identified for SIDS (e.g., prevention of prone sleeping, overheating, and exposure to cigarette smoke); and 2000–2012 to determine if introduction of Hib or pneumococcal vaccines had an effect on SIDS. There were reductions in SIDS cases identified following introduction of the Hib vaccine in 1999, introduction of the earlier immunization schedule in 2006, and introduction of immunization for pneumococcus in 2008. While reduction of prone sleeping or overheating cannot be ruled out as contributing to these decreases, the proportion of mothers who smoked remained constant. The evidence for infection/inflammation in explained deaths declined significantly over the three periods studied. Evidence of inflammation/infection in SIDS cases remained steady. Initiation of infant immunization in the United Kingdom at 2 months was associated with a shift in the age ranges of SIDS infants (7, 8), and although the numbers were small, there was a similar pattern of a sharp decline among infants who died at 3 months of age.

According to a recent study, the component of the postmortem examination that was the most helpful in diagnosis was the histological examination, followed by macroscopic examination, microbiological investigations, and clinical history.

The majority of infection-related diagnoses were identified primarily by histological sampling rather than microbiological analyses, although microbiology aided in a diagnosis for 20% of cases that would have otherwise gone undetected (14). While autopsy findings have varied, signs of inflammation and responses to infection have appeared out of proportion to preexisting symptoms. The increase in inflammatory markers found in SIDS infants indicates that infection and inflammation might contribute to some of these deaths, either as a direct cause or the trigger of a lethal event. It might also be indicative of vulnerability in the immune response to an infectious trigger in infants. The protective effects of immunization (18, 19) noted for SIDS is further evidence for infection/inflammation playing a role in these infant deaths.

Author Contributions

Each of the authors made substantial contributions to the conception, design, analyses, and interpretations of the work. They assisted in preparing the article, critically assessed the final version, and agree to be accountable for the accuracy and integrity of the work.

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REVIEW
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Escherichia coli and sudden infant death syndrome

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This review examines the association of strains of *Escherichia coli* with sudden infant death syndrome (SIDS) and the possible role these bacteria play in this enigmatic condition. The review addresses evidence for *E. coli* in SIDS infants, potential sources of *E. coli* in the environment, colonization by commensal and pathogenic strains, the variety of currently accepted pathotypes, and how these pathotypes could compromise intestinal integrity and induce inflammation. Both intestinal and extraintestinal pathotypes are compared in relation to the apparent liability in which virulence traits can be gained or lost by strains of *E. coli*. The way in which *E. coli* infections fit with current views on infant sleeping position and other SIDS risk factors is highlighted.

Keywords: sudden infant death syndrome, SIDS, *Escherichia coli*

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Commensal and Pathogenic *Escherichia coli*

While *Escherichia coli* and "coliform" bacteria are considered part of the normal microbiome of the human intestinal tract, they have been identified in studies of infants who died suddenly and unexpectedly, infants classified either as sudden infant death syndrome (SIDS) and more recently sudden unexpected death in infancy (SUDI) (1–3). When confined to the gut, these bacteria are generally considered harmless; however, a number of pathotypes of *E. coli* can cause disease in humans and animals. The pathotypes are characterized by their ability to produce certain virulence factors, toxins and adhesins. They include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAgEC), Shigatoxigenic *E. coli* (STEC) [verotoxigenic *E. coli* (VTEC)], enteroinvasive *E. coli* (EIEC), and diffuse adherent *E. coli* (DAEC). Their characteristics will be considered in more detail below.

All *E. coli* are not the Same

Until 1940s, pathogenic *E. coli* were not identified; they could not be distinguished by techniques available from non-pathogenic commensal *E. coli*. The pioneering work of Kauffmann in developing a serotyping scheme enabled strains belonging to certain O:H serotypes to be linked to certain of the pathotypes. For many years, EPEC was only recognized by serotypes until specific virulence factors were identified.

Mechanisms by which the pathogenic *E. coli* gain access to and remain within a susceptible host were unknown but considered likely to be similar to that of commensal *E. coli* over extended periods of co-evolution. Studies on commensal *E. coli* in 1970s provided a basis for understanding the colonization of the human body by *E. coli*.

Commensal Strains

In the first of these studies (4), the complete feces from 9 healthy adults were examined with 10 sites on each specimen assessed and at least 10 *E. coli*-like colonies collected from each site. These revealed

a great diversity of types, although in all stools except 1 a single predominant type was present at all 10 sites. This indicated that generally when an *E. coli* isolate is selected from a fecal specimen, it is likely to be the predominant type, but minor types might be missed.

An extensive study on neonates' acquisition of *E. coli* (5) found "*E. coli* are present in the vagina of women and that the acquisition of these *E. coli* by babies is related to the length of time that the birth takes. It was also noted that there is a relationship between the *E. coli* found in the feces of the mothers, the mucus swallowed by the babies at birth, and subsequently in the feces of the babies'. While this might be how infants acquire their *E. coli* and other microflora, it was noted that "Caesarian section babies are generally not likely to become colonized by their mothers' fecal *E. coli*." They were, however, colonized as rapidly as vaginally delivered babies. These studies showed that the babies' gut was colonized early and these colonized babies became the foci for spread of *E. coli* to other babies. Environmental studies indicated that "contaminated hands and uniforms of the nursing staff may be the main vector for transmitting *E. coli*." It was also noted that these *E. coli* acquired or lost certain traits during their spread including antibiotic resistance, serological variation, and even biochemical characteristics (6). Spread of *E. coli* was to a maximum of four other infants; Caesarian babies were particularly prone to colonization by these types of *E. coli*.

Pathotypes

Studies on commensal *E. coli* summarized above were in part inspired by two earlier studies. The first was initiated as a result of a survey on *E. coli* urinary-tract infection (UTI) among patients who had been admitted to a female general medical ward over a period of 2 years (7). The use of an extensive set of *E. coli* typing antisera enabled a more complete identification and analysis. It was noted that the O types responsible for most of the infections belonged to relatively few common types.

Patients with diabetes, malignant disorders, rheumatoid arthritis, and hypertension had a much higher incidence of *E. coli* UTI than the rest of the ward population. In addition, there were small epidemics of UTI due to specific *E. coli* O types in the ward. These studies showed that none of these was acquired by catheterization or instrumentation. The evidence accorded with an oral transmission of type-specific *E. coli* from the intestinal reservoirs of human carriers.

Among 20 patients (8) who had previously been free of UTI and who developed infection, 13 developed these infections within 12 days of gynecological operations accompanied by insertion of a self-retaining catheter. The infections were caused by a type, which had been present before the operation in the rectum, vagina, or both.

These studies indicate that commensal *E. coli* can be transmitted among infants and individuals who are susceptible can acquire such *E. coli*, observations that could be relevant to susceptibility of both neonates (especially premature) and women who developed UTI.

Investigations carried under the sponsorship of the military authorities examined the cause of travelers' diarrhea, which affects individuals newly arrived in a foreign country, usually within 14 days of arrival (9). A group of 540 British soldiers traveled by air from England to Aden; of these 38 had an episode of diarrhea during their first 14 days after arrival. Fecal specimens from 35 were investigated and 2 yielded strains of *Salmonella* spp., no known pathogens were isolated from the remaining 33. A new serotype of *E. coli* (O148.H28) was isolated in the acute phase from 19 of these cases. In the remaining 14 (40%) cases, a number of different *E. coli* serotypes were isolated. These included serotypes associated with infantile diarrhea and not related to travelers' diarrhea. The peak of the isolations of *E. coli* O148:H28 corresponded with the peak incidence of travelers' diarrhea. This serotype was never isolated from a healthy subject. A year later, in a laboratory in England, a technician working with this serotype developed a severe episode of diarrhea and *E. coli* O148:H28 was recovered in pure culture from his stools. The same serotype as well as O6:H16 were subsequently isolated from American soldiers in Vietnam (10).

Since these early discoveries at least 78 detectable O antigens and 34 H antigens associated with ETEC have been described. Among the most common serotypes, which produce either the so-called heat stable enterotoxin (ST), heat labile enterotoxin (LT), or both are O6:H16 (LT/ST), O8:H9 (ST only), O25:NM (LT only), O78:H12 (ST only), O148:H28 (ST only), and O153:H45 (ST only). Their prevalence and phenotypes [based on the toxins they carry or the colonization factors (CFs) they express] can vary depending on the location. Many of these serotypes were also commonly isolated from environmental sources and human cases and outbreaks around the world (11). These studies confirmed that *E. coli* can be true pathogens as well as commensal and they can be acquired and shed by humans.

In 1983, a mild outbreak of diarrhea occurred in the neonatal ward (12), in which the earlier studies on the spread of commensal *E. coli* had been carried out (5, 6). Despite introduction of strict control measures, the epidemic strain (O125:K70:H21) spread to 16 infants while the commensal strains spread maximally to 4 infants, similar to the spread observed in the previous study for which no specific containment had been undertaken. Commensal *E. coli* spread to other infants unimpeded and the outbreak strain spread far more than the commensals.

Initially, *EPEC* was identified on the basis that certain serotypes were regularly associated with infantile gastroenteritis, but the actual virulence factors were not known. Following discovery of the virulence factors, they were shown to be present not only in the currently isolated EPEC strains but also in original archival EPEC strains, isolated long before the description of these virulence factors (13) demonstrating that EPEC comprised a specific lineage of pathogenic *E. coli*.

Shigatoxigenic *E. coli* or *VTEC* are another pathotype first described in 1977 (14) in a study of the effects of culture supernatants from various *E. coli* on different cell lines including Vero cells (14). While a number of different serotypes were identified as being associated with these pathotypes (15) one serotype (O157:H7), first described in 1983 (16) has dominated the STEC/VTEC literature. It was soon realized that the Verotoxins were similar to the Shiga toxin associated with strains of *Shigella dysenteriae*, which had been described as long ago as 1903 (17, 18). Non-O157 STEC/VTEC was generally overlooked

in the early years of STEC/VTEC research, but their importance as causes of sporadic and outbreak disease is now well established (15).

It has been shown over the years that various "mobile genetic elements," such as transposons, insertion sequences, bacteriophages, and plasmids, can exist either integrated into the chromosome or through self-replication within the new host to provide new traits and fitness advantages. Most definable virulence factors found in pathogenic *E. coli* are derived from genetic mobile elements. For example, most of the genes for toxins and CFs required for the pathogenesis of ETEC are found almost exclusively on plasmids (11). These plasmids can move between strains supplying a variety of palettes of virulence factors. A recent example of this type of interaction was shown with the mixing of virulence factors, resulting in one of the most serious outbreaks caused by *E. coli*. This was the outbreak in 2011 (19) due to enteroaggregative *E. coli* O104:H4 strains, which were also able to produce Shiga toxins and which affected around 4000 individuals in Germany and many more in 16 other countries. Recently, it was shown that the enteroaggregative strain had acquired the bacteriophage coding for Shiga toxin 2a in cattle (20). Most *E. coli* infections are enteric. Extraintestinal infections, such as UTI and neonatal meningitis, probably, also have an enteric origin (7, 8, 21). This could have implications in regards to SIDS (*vide infra)*.

Escherichia coli and SIDS

Sudden infant death syndrome remains a major cause of death of infants under 1 year of age. It was defined as long ago as 1969 (22), and its cause remains enigmatic. A large number of studies by both us and others have suggested that a bacterial infection may be a contributing or causal factor (23–30). *E. coli* is often identified in these infants. α-Hemloysin (HLyA)-producing serotypes of *E. coli* causing urinary tract and other extraintestinal infections are more commonly isolated from SIDS infants (29). These include O1:H$^-$, O1:H5, O2:H1, O4:H5, O6:H1, O25:H1, and O75:H5 (31). A recent study (32) found that the α-HLyA of *E. coli* triggers intestinal inflammation in mouse models of inflammatory bowel disease by impairing the intestinal barrier thereby intensifying antigen uptake. α-HLyA-producing *E. coli* were more commonly isolated from the human mucosa in patients with active ulcerative colitis than in controls. α-hemolysin-producing *E. coli* needs to be considered in SIDS. The effects of HLyA and/or Ehly on the enterocyte and gut wall integrity could result in mucosal perturbation or damage promoting translocation of bacteria (*E. coli* or other gut microbiota) into the bloodstream either through induced inflammation or directly [see below and the accompanying article by Goldwater (33)].

The α-hemolysin is synthesized as a 1024-amino acid polypeptide, and then intracellularly activated by specific fatty acylation. A second activation step takes place in the extracellular medium through binding of Ca^{2+} ions (Ca^{2+} HlyA) (34). Recent studies have further shown that α-HLyA of *E. coli* interacts directly with cholesterol (35). These studies note that the insertion of α-HLyA is favored in cholesterol- and sphingomyelin-containing membranes. These studies, which demonstrated a clear interaction between α-HLyA and cholesterol, show this favors a conformational change of the α-HLyA that permits its correct insertion into the membrane to form membrane pores. Some strains of *E. coli* produce a different hemolysin from α-hemolysin, i.e., enterohemolysin (Ehx or Ehly), which is especially associated with STEC, although not all STEC produce it. There are enterohemolysin-producing *E. coli* that do not produce the Shiga toxins (36).

Enterohemolysin was shown to be a toxin and a member of the RTX (repeats-in-toxin) family of toxins. In its free form, it is lytic toward human endothelial and intestinal epithelial cells. It is also associated with outer membrane vesicles (OMV). The role of these OMVs was largely unknown (37). Their investigation (37) involved microscopic, biochemical, flow cytometry, and functional analyses of human brain microvascular endothelial cells (HBMEC) and Caco-2 to examine the role of OMV-associated toxins. These demonstrated that OMV-associated enterhemolysin does not lyse the target cells but triggers their apoptosis. Following internalization of the OMV-associated enterhemolysin by HBMEC and Caco-2 cells, a cascade ensues, which lead to apoptotic cell death as demonstrated by DNA fragmentation and chromatin condensation in the intoxicated cells. This ability of OMV-associated enterohemolysin to trigger the mitochondrial apoptotic pathway in human microvascular endothelial and intestinal cells is considered to be a newly recognized mechanism for a bacterial toxin to enter host cells in order to target mitochondria. As far as is known, the production of enterolysin by SIDS-associated *E. coli* has not been fully investigated; however, we have observed Ehly to be common in SIDS strains (unpublished). A rapid test for the production of enterohemolysin by *E. coli* is available (38). STEC has been identified in bovine feces and non-shiga-toxigenic *E. coli*, which belong to typical STEC serotypes; these carry most of the STEC virulence factors apart from being Shiga toxigenic in many cases also produce enterohemolysin (36). Such *E. coli* are known as atypical enteropathogenic *E. coli* (aEPEC) and have also been found to be present in patients with STEC infections and it appears that the acquisition or loss of the phage coding for the production of Shiga toxin in patients infected with STEC (39) is a not uncommon phenomenon. This was first described in 2005 (40).

It has been demonstrated (41) that certain ETEC that cause travelers' diarrhea produce a protein, EatA, a plasmid-encoded serine protease, which degrades MUC2, which is a major protein present in the mucus layer of the small intestine. The removal of MUC2 accelerates the access of the ETEC enterotoxins to the enterocyte surface. While this has not been sought in SIDS-associated *E. coli*, the fact that some of these strains can produce this factor does not preclude others also producing this or similar factors. Other studies (42) show that another factor, YghJ, is highly conserved in ETEC and is a metalloprotease that degrades the major mucins, MUC2 and MUC3. This metalloprotease has also been found to be present in other *E. coli*, including the enteroaggregative *E. coli* O104:H4 and even strains of *Vibrio cholerae*. These have not yet been investigated in SIDS.

Of relevance is a study in baboons on acute respiratory distress syndrome (ARDS) (43), which mimics *E. coli* sepsis in humans. The baboon model displays the early inflammatory phase with extensive necrosis. If certain *E. coli* can cause ARDS, then it is

possible that a similar mechanism occurs in SIDS given that the clinical and pathological findings in SIDS babies reflect a process consistent with sepsis and/or toxemic shock. This is supported by sterile site evidence of bacteremia (33, 44, 45) and the knowledge that bacteremia is a profound inducer of hypoxemia (thought to be involved in the final pathway). This model is supported by clinical evidence of fever [sweat-soaked bedding (46) and raised rectal temperature (47) and the pathological findings of intrathoracic petechiae, heavy wet lungs, and liquid heart blood and elevated fibrin degradation products (48, 49)], findings compatible with a process of bacterial sepsis. The physiological findings of hypoxemia and bradycardia and asystole observed in monitored babies dying of SIDS (50), again point to bacterial sepsis or toxemia as the final event in SIDS.

Unfortunately, systematic studies have not been performed where isolates of *E. coli* obtained from cases of SIDS and from healthy infants have been subjected to a full battery of all the possible toxins and virulence factors that are known to be produced by *E. coli*. Perhaps, such systematic studies will be undertaken in the future. Direct toxin identification studies might be possible in body fluids, such as urine, in which kidneys concentrate or filter circulating bacterial toxins from the bloodstream. Preliminary unpublished data indicate urine to be a useful fluid for toxin detection utilizing advanced proteomic techniques (Morris, personal communication). This work could provide the breakthrough that has been too long awaited by SIDS researchers.

SIDS Risk Factors in Relation to Acquisition of *E. coli*

Bed Sharing

An association between bed sharing and SIDS has been noted (51, 52); however, why bed sharing occurred on the occasion of the last sleep has not always been clearly defined. A recent study (53) systematically reviewed the issue but only partially answered such questions as Was the child unsettled/unwell which led to the baby joining the parents in their bed? The review divided reasons for bed sharing into either "intentional" or "reactive;" "monitoring" the baby was given as a reason in 56.9% of situations (66.7% in Blacks) and "crying" in 32.4%. These results could indicate that SIDS babies were unwell on the last sleep. Clearly, further refinement in reasons for bed sharing and the interactions with other environmental factors is needed. Babies of families that regularly bedshare seem to be at low risk of SIDS (54). Also, the effect of parental drug/alcohol consumption has been taken to imply that this would lead to overlaying. An alternative hypothesis would be that drug-affected adults might not be alerted to an unwell/unsettled baby. An even more basic suggestion is that the infants acquire *E. coli* flora from close contact with parents or their bed linen.

While such questions remain unanswered, it is generally suggested that parents should share the room but not the bed with the infant (55, 56). An extensive study was undertaken in USA. to determine those trends and factors that might be involved with bed sharing over a period from 1993 to 2010 (57). This study, which was conducted through approximately 1000 telephone interviews annually, showed that there was a steady increase in bed sharing throughout the period of study. A further study suggested that breastfeeding might be affected by bed sharing (58) and *vice versa* (53). In another study (59), the authors identified as "three areas of concern" being (a) the baby sleeping in areas other than crib or bassinet, (b) bed sharing, and (c) non-supine sleeping.

Mode of Delivery

A number of environmental factors have been investigated with regard to acquisition of bacteria by neonates (4–6). Mode of delivery (Cesarean or vaginal delivery) being important. There are indications that bacterial characteristics also play an important role in dissemination, acquisition, and successful colonization (12).

Used Cot Mattresses

The association between used cot mattresses and SIDS (60) was investigated by assessment of used polyurethane foam cot mattresses. The bacterial population density was greatly increased in used mattresses. *E. coli* was isolated from the dorso region of the mattresses, significantly so if the infant slept in the prone position, another significant risk factor for SIDS (61).

SIDS Risk Factors in Relation to Acquisition of and Effects of *E. coli*

Viral Respiratory Infections

Experimental studies have indicated that mild virus infection might potentiate the inflammatory response to bacterial toxins, particularly endotoxins. Moscovis et al. (this issue) (62) demonstrated in a model system that interferon-γ (IFN-γ) significantly enhanced pro-inflammatory response to endotoxin and significantly reduced the anti-inflammatory IL-10 response. The effects of IFN-γ on responses to the soluble *E. coli* toxins have not been examined.

Environmental Pollutants

A recent preliminary study (63) showed that the intestinal bacterial flora of neonates is influenced by house dust. Using high through-put sequence analysis of portions of the16S rRNA gene, it was found that despite significant differences between the dust and fecal microbiota as revealed by non-metric multidimensional scaling (NMDS) analysis, permutation analysis confirmed that 14 bacterial operational taxonomic units (OTUs) representing the classes Actinobacteria, Bacilli, Clostridia, and Gammaproteobacteria co-occurred at a significantly higher frequency in matched dust–stool pairs than in randomly permuted pairs, indicating an association between these dust and stool communities.

A study (64) of house dust and its relation to the skin surface swab samples of occupants in four homes were analyzed for their bacterial content using more accurate culture-independent methodology. Species-level operational taxonomic units (SLOTUs) were used to compare the results. Phylogenetic description of SLOTUs of the bacterial sequences was analysed from the different house dusts and skin surface swabs, which represented random samples of bacteria present in a given sample. The study (65) showed that the presence of bacterial endotoxins in house dust and investigated the role that they may play in the

development of allergen sensitization. As LPS is also a powerful inducer of inflammatory responses, these findings require more investigation.

A preliminary investigation of the gut micro flora in SIDS compared the intestinal contents from 52 SIDS cases, with 102 fecal samples from age-matched live comparison infants (66). These were screened by PCR to target 16s RNA genes of *Clostridium innocuum*, *Clostridium perfringens*, *Clostridium difficile*, *Bacteroides thetaiotaomicron*, and *Staphylococcus aureus.* While *E. coli* were not included in these studies, the distribution of the test microorganisms can be used to extrapolate the possible roles of *E. coli*. The paper showed that the gut microbiome of SIDS babies differs from that of normal babies. The major finding was significantly more babies dying prone were colonized by *S. aureus* colonization than babies similarly colonized but dying in other positions (supine/side) (odds ratio = 20.25; CI = 3.60–134.92). This supports the hypothesis that prone sleep position increases the risk of such colonization by *S. aureus* and *E. coli* (61) or induction of temperature dependent toxins, such as the staphylococcal pyrogenic toxin (67), as the nasal temperature of infants in the prone position is significantly raised. There was a highly significant association between pronesleeping in both gut and a sterile site infection (OR = ∞; CI = 2.04–∞). There is a large body of evidence that bacterial infection plays a role in the events triggering SIDS. [Many of the pathology findings are compatible with a process of bacterial sepsis together with the physiological findings of hypoxemia and bradyasystole followed by gasping, (50) point to bacterial sepsis/toxemia as the final event in SIDS pathogenesis.]

E. coli in SIDS and Inflammation

A history of diarrheal illness in the last week of life is a frequent finding in SIDS (46). Nelson et al. (54) supports the probability of the occurrence of gut inflammatory changes in SIDS. In general inflammation of the gut in SIDS has not been widely studied, but supportive evidence of compatible histological changes has been published (68) and examination of autopsy reports frequently shows hyperplasia of germinal centers and follicular hyperplasia of mesenteric lymph nodes.

A recent study (69) showed that infection by enteric pathogens, such as *Salmonella enterica* serovar Typhimurium (S. Tm), is antagonized by a highly complex intestinal microbiome, i.e., colonization resistance. Disruption of colonization resistance by such conditions as antibiotic therapy, a germfree state or an immature microbiome of low complexity results in increased susceptibility to oral infection with pathogens of the *Enterobacteriaceae* family (70–72). Pathogen (e.g., S. Tm)-induced gut inflammation promotes parallel blooms of S. Tm and host commensal *E. coli*. These blooms out-compete residual microbiota with *E. coli* and other *Enterobacteriaceae* taking advantage of the inflammatory conditions. The bacteria compete successfully for resources by directly antagonizing competitors by producing antimicrobials including bacteriocins. The bacteriocins produced by *Enterobacteriaceae* are colicins. Blooms of commensal and pathogenic *Enterobacteriaceae* exploit the abundant conditions provided by the inflamed gut (73–75). In man, up to 15 different *E. coli* strains can be detected in the gut microbiome (72). The success of the inflammation-induced enterobacterial bloom has been attributed to colicins, such as ColIb (cib), which provides effective defense against competing bacteria. Expression of cib and CirA (the cognate outer membrane receptor of cib on colicin-sensitive *E. coli*), is regulated by iron limitation. In the context of SIDS, and on the basis of epidemiological evidence of diarrheal symptoms in the days preceding death, and the sterile site data (33, 44, 45) it is plausible that invoked inflammation of the gut could lead to translocation of bacteria from the gut lumen into the portal and systemic blood stream and thence provide the stimulus for a lethal cytokine storm. It is clear that detailed analysis of the gut in SIDS and the microbiome within are neglected areas of research. In time, this approach might provide important clues to the SIDS enigma. If the studies by Gómez-Moreno et al. (76) are shown to be universally applicable then testing fecal specimens for the several genes of bacterial origin, which had been previously associated with inflammation may well be seen as risk factors for SIDS and treatment to remove these bacteria may well be achievable. Until such studies (76) are universally undertaken as part of the forensic investigation into the sudden death of an infant, full conclusions cannot be drawn.

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REVIEW
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Gut microbiota and immunity: possible role in sudden infant death syndrome

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The gut microbiome influences the development of the immune system of young mammals; the establishment of a normal gut microbiome is thought to be important for the health of the infant during its early development. As the role of bacteria in the causation of sudden infant death syndrome (SIDS) is backed by strong evidence, the balance between host immunity and potential bacterial pathogens is likely to be pivotal. Bacterial colonization of the infant colon is influenced by age, mode of delivery, diet, environment, and antibiotic exposure. The gut microbiome influences several systems including gut integrity and development of the immune system; therefore, gut microflora could be important in protection against bacteria and/or their toxins identified in SIDS infants. The aims of the review are to explore (1) the role of the gut microbiome in relation to the developmentally critical period in which most SIDS cases occur; (2) the mechanisms by which the gut microbiome might induce inflammation resulting in transit of bacteria from the lumen into the bloodstream; and (3) assessment of the clinical, physiological, pathological, and microbiological evidence for bacteremia leading to the final events in SIDS pathogenesis.

Keywords: sudden infant death syndrome, gut, microbiome, immunity

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Introduction

The common bacterial hypothesis (1, 2) and the role of bacteria in the causation of sudden infant death syndrome (SIDS) have not been considered in depth by mainstream researchers nor has there been broad interest in the potential contribution of infection and inflammation to these deaths. Studies on the microbiology of SIDS (3–7) provided explanations for SIDS risk factors and potential mechanisms in which inflammatory responses could affect abnormal arousal, respiration (8, 9), and/or brain stem compromise (10); these are areas that have preoccupied the mainstream of SIDS research for decades without successfully providing hypotheses congruent with epidemiological and pathological features of SIDS.

The infection model of SIDS stands on substantial evidence identifying: *Staphylococcus aureus* and its enterotoxins (11–14); toxigenic *Escherichia coli* (15, 16); *Clostridium perfringens* (3, 4); and recent findings (based on a restricted number of bacterial species identified by culture and PCR) of significant differences in the gut microbiome between SIDS and healthy babies (17). A review of the gut microbiome of babies in the context of immunity and immune/inflammatory responses to bacterial infection was considered timely: to help clarify the role of gut/mucosal immunity in relation to SIDS; to explain the apparent mucosal dysregulation reported for one SIDS infant (18). The basis for a link between the gut microbiome and SIDS is founded upon published evidence that provides a

logical explanation of underlying pathomechanisms involved in SIDS. The evidence is summarized in **Table 1** and addressed later in the review.

Background

It has been known since 1905 that the microbiota of neonates undergo change with growth and development (19). The type of early microbiota is important in terms of development of the immune system, and this could influence susceptibility to infection, induction of gut inflammation, and adverse outcomes of infection. Improved understanding of how the gut microbiota and type of feeding affect immune and neurodevelopment has arisen since the introduction of molecular techniques to identify and quantify bacterial genera and species involved (17, 20–22).

A literature search reviewed recent publications covering development of the infant gut microbiota in conjunction with development of the infant immune system and related functions. Articles pertaining to the gut microbiota and SIDS/sudden unexplained infant deaths (SUDI) were also reviewed. Search engines used included PubMed, MedlineRanker, PubCrawler, Google Scholar, and Open Access Library (OALib).

TABLE 1 | Comparison between findings in SIDS compared to sepsis.

	SIDS	Sepsis
Pathological findings	Vasculopathy (intra-thoracic petechial hemorrhages)	Vasculopathy
	Coagulopathy (raised FDPs)	Coagulopathy (raised FDPs)
	Heavy, wet, congested lungs	Heavy, wet, congested lungs
	Renal shutdown (empty bladder)	Renal shutdown
	Evidence of recent pro-inflammatory cytokine release	Evidence of recent pro-inflammatory cytokine release
	Cerebro-spinal microgliosis	?Cerebro-spinal microgliosis
	Vasculopathy	Vasculopathy
	Coagulopathy	Coagulopathy
	Raised CSF IL-6	
	Raised rectal temperature (fever)	Raised temperature (fever)
Clinical findings	Sweatiness (fever)	Fever
	Recent gastrointestinal or respiratory viral infection	Underlying infection
Physiological findings	Hypoxemia, tachycardia then bradycardia, asystole, gasping, death	Hypoxemia, tachycardia then bradycardia, asystole, gasping, death
	Raised rectal temperature	Fever
Microbiological findings	Normally sterile site infection	Normally sterile site infection
	Evidence of bacteremia	Bacteremia
Risk factors	Genetic (various; immune gene polymorphisms)	Genetic (various)
	Prenatal (exposure to smoke products)	Prenatal (various)
	Postnatal (exposure to smoke products, prone sleep, etc.)	Postnatal (smoking, immunopathy)

?, undetermined.

Role of Gut Microbiome During the Critical Developmental Period Associated with SIDS

Colonization

Bacterial colonization of the human infant colon is influenced by many factors – age, mode of delivery (23), diet, environment, and antibiotic exposure (20, 21, 24–26).

There is growing evidence that cesarean section (CS) (not regarded as a SIDS risk factor when controlled for gestational age) (27) is linked to an impoverishment of natural development of the immune system. Exposure of the neonatal gut to bacterial priming (as occurs with vaginal delivery) appears to be missed in babies born via CS (28). One of the main associations with CS is low gestational age. Prematurity remains a significant and largely unexplained risk factor for SIDS; the vulnerable, immature host notwithstanding (29). It is presumed that the abovementioned mechanisms (27) over and above the vulnerable host would go some way to explain this increased risk.

Diet influences the gut microbiota. The effect of breast milk on the infant immune system is considered to derive benefit via its effect on gut bacterial colonization (30). Breast feeding has a protective effect against SIDS and given the effects on the gut microbiota, suggests that these bacteria might play a role in addition to maternally transferred cellular and humoral immunity. CS remains the most common mode of delivery of preterm infants. The possibility that these babies will receive breast milk or enteral feeds remains remote while the opposite is true for receiving treatment with antibiotics. Animal studies show that a lack of enteral nutrition may be associated with an increased risk of septic shock due to bacterial translocation caused by intestinal epithelial cell apoptosis (31). These factors thus adversely affect preterm infants in terms of their susceptibility to infection and to inflammatory gut disease such as necrotizing enterocolitis (NEC) (30). Because SIDS infants are more likely to have had symptoms of infectious diseases in the last week or the last day before death, they are more often examined by a physician and given antibiotics than control babies (32). It remains unknown if antibiotics specifically contribute to the risk of SIDS.

Gut Immunology and Homeostasis

The intestinal immune system is modulated in response to environmental factors shortly after birth (33). Battersby and Gibbons (34) summarized the emerging knowledge of how the gut maintains immune homeostasis with non-pathogenic bacteria (34). A better understanding of the molecular and cellular mechanisms sustaining homeostasis is emerging (35–37).

Mechanisms by Which the Gut Microbiome Could Induce Inflammation and Transit of Bacteria to the Blood Stream

Microbial Recognition and Inflammation

The nuclear factor kappa B (NF-κB) pathway is responsible for microbial recognition and inflammatory responses in the adult

gut. NF-κB, the so-called "master switch" of the immune system, has numerous roles in innate and adaptive immune responses and inflammation. In the mature gut, pathogen-activated molecular patterns (PAMP)-specific region of a gut pathogen binds to its corresponding Toll-like receptor (TLR) on an enterocyte (EC) and causes release of NF-κB (38). The reaction enters the nucleus where genes mediating inflammation are turned on (38–40). In response to bacterial lipopolysaccharide (LPS), fetal ECs upregulate the NF-κB pathway and produce more of the chemokines, CXCL2 and CXCL8 (41).

Tolerance and Commensal Bacteria

First exposure of the neonatal gut to LPS and antigens from non-pathogenic commensals may result in significant inflammatory responses. The mechanisms by which tolerance to non-pathogenic commensals is established at this most critical period require further understanding. Part of the process includes inhibition of the NF-κB pathway by commensal bacteria (40, 42).

As ECs mature, expression of surface TLRs reduces, as do downstream signaling complexes, while inhibitory factor kappa (iκB), a negative regulator, increases. This appears to provide "protection" without adverse inflammation during colonization (43). Alterations in innate immune response genes in fetal ECs contribute to NEC (44) and suggest that an inappropriate inflammatory response takes place in premature babies. Commensal bacteria exert a variety of effects on cytokine responses. These include production of pro-inflammatory cytokines via the IL-25–IL-23–IL-17 axis (45), possible downregulation of IL-17 in Th-17 cells resulting in diminished inflammation, and upregulation of IL-25. IL-25 is thought to suppress IL-23 by gut dendritic cells (DCs) with subsequent diminished IL-17 and an anti-inflammatory effect (45). Commensal bacteria also affect responses of CD103+ DCs to products of ECs (such as retinoic acid, TDF-β, and cytokine thymic stromal lymphopoietin (TSLP) that effectively dampen the adaptive immune response in those (46). In addition, commensal bacteria (such as *Bifidobacterium* sp.) may upregulate IL-10 production by DCs (47).

Pathogen Recognition

Pathogen recognition by innate immune cells initiates an immune response to infection. Key PAMPs instigate effector responses through activation of pathogen pattern recognition receptors (PRRs), which include well-characterized TLRs. Adults and children carry an increased infection risk if they carry TLR polymorphisms. The same is true for SIDS (48). Neonatal infection with Gram-positive and Gram-negative bacterial infections is associated with enhanced expression of TLR2 and TLR4 (38). Dysregulation of TLR4 expression is associated with development of NEC (49), commonly associated with neonatal sepsis. In addition to activation by exogenous PAMPs such as LPS or viral ssDNA, TLRs occurs through endogenous damage- or danger-associated molecular patterns (DAMPs), intracellular proteins, and inflammatory mediators released by damaged or apoptotic cells. These include endogenous Alarmin High-Mobility Group Box 1 (HMGB1), heat shock proteins, and uric acid; each contributes to the pathophysiology of septic shock. Dysregulated HMGB1 expression associated with progression of sepsis to septic shock (43, 50) perpetuates the inflammatory response. Disruption of EC tight junctions also occurs and leads to increased bacterial translocation (51) and bacteremia.

Recognition of PAMPS or DAMPS by PRRs results in activation of NFkB resulting in pro-inflammatory cytokine and chemokine production and induction of the IRF transcription factors that mediate production of type I interferon. The NOD-like receptor (NLR) family of proteins shares a number of common domains and many are involved in PAMP and DAMP sensing. The process results in NFkB activation and inflammatory gene expression (52, 53). NLRP proteins oligomerize directly or indirectly with caspase1 through the caspase recruitment domain (CARD) to form an inflammasome. Inflammasomes are essentially caspase-activating complexes.

The anti-inflammatory nature of IL-10 is well known, except, perhaps in SIDS where the cytokine might contribute to a fatally defective pathogen recognition (48). In the neonatal gut, IL-10 is reported to reduce inflammation as evidenced by inhibition of key parts of the unfolded protein response (UPR) (38, 54). Paneth and goblet cells respond to abnormal protein handling with the UPR, which results in local tissue inflammation through activation of immune cells including neutrophils (55).

Role of Innate Lymphoid Cells

These cells do not possess a specific antigen receptor; however, innate lymphoid cells (ILCs) are able to secrete a number of cytokines equivalent to those produced by the subsets (subsets T_H2, T_H17, and T_H22.) of T helper cells. Subsets of ILCs have been demonstrated. ILC1 cells differ from natural killer (NK) cells in that they lack CD56, CD16, and CD94 NK cell markers as well as perforin, and granzyme B. ILCs function in lymphoid organogenesis, tissue remodeling, antimicrobial immunity, and inflammation, particularly at barrier surfaces (56). Their ability to respond promptly to insults inflicted by stress-causing microbes strongly suggests that ILCs are critical in first-line immunological defenses. A number of families of ILCs have been described. These include Rorγt-expressing cells involved in lymphoid tissue formation, mucosal immunity, and inflammation. Type 2 ILCs are important for helminth immunity, type 3 for mucosal integrity and healing.

Gut homeostasis depends on minimizing responses to commensal bacteria, which can lead to inflammatory bowel disease (IBD) in genetically predisposed individuals; but there is a need to retain the ability to recognize and control the growth of infectious pathogens (56). Group 3 innate lymphoid cells (ILC3) help maintain intestinal homeostasis by producing the cytokine IL-22, which promotes mucosal healing and maintains barrier integrity. Microbial signals trigger production of IL-23 and IL-1β; these stimulate ILC3s to produce IL-22, leading to induction of antibacterial peptides and epithelial cell regeneration (57). The cell type producing IL-23 in response to microbial signals is unclear and much debated; resident mononuclear phagocytes (MNPs), inflammatory monocytes, and conventional DCs have all been implicated. Longman et al. (57) provide a clue. In mice with *Clostridium rodentium* infection and in patients with colitis, CX_3CR1^+ MNPs are superior producers of IL-23 and IL-1β, and they are very efficient in inducing IL-22 production by ILC3 (56, 58).

The development of Peyer's patches and ILC2 and ILC3 subsets depends on *Nfil3 (nuclear factor, interleukin 3-regulated)*. Loss of *Nfil3* selectively decreases Peyer's patch formation and is associated with defective recruitment and distribution of ILCs within the patches. ILC subsets strongly express *Nfil3*. Deletion of *Nfil3 genes* adversely affects development of all subsets, so that *Nfil3*$^{-/-}$ knockout mice show increased susceptibility to infection or pro-inflammatory agents confirming the importance of the role of *Nfil3* in development of ILC subsets upon which the gut depends for protective immunity (59), especially against intestinal pathogens (60). Additionally, development of all innate lymphoid cell subsets depends on Nfil3.

Gut Microbiome, Inflammation, and SIDS

The microbiome contributes to development and sustenance of the immune system. This includes protective immune effector function in the healthy host as well as in cases of disease. It is noted that SIDS cases frequently have been unwell in the days leading up to the death. Diarrheal symptoms are often reported (61). Bacteremia associated with viral gastroenteritis often with an accompanying fever is a known complication (62). Bacteremia without localizing source is also well known (63), and occult bacteremia is not uncommon in infancy (64). Such episodes of bacteremia are usually benign, indicating that most babies' immune responses are able to cope without lethal consequences. By contrast, SIDS babies might have dysregulated responses (48). In these circumstances, it could be suggested that bacteremia could result in an overwhelming cytokine storm with consequent sepsis/toxemia resulting in the baby's demise.

In addition to the pathogenic *E. coli* groups (Bettelheim and Goldwater, this issue), two groups of the gut microbiome have been investigated in relation to SIDS – *Bacteroides thetaiotaomicron* and *Clostridium* species.

Gram-Negative Bacteria and Inflammation

It is known that the NLRC4 inflammasome is activated through caspase1 by Gram-negative bacteria containing type III or type IV secretion systems, e.g., *Salmonella*, *Shigella*, *Legionella*, *Pseudomonas*, *Yersinia*, and some *E. coli*. NLRC4 specifically recognizes flagellin; consequently, non-motile strains are unable to activate caspase1. Flagellin protein alone has been shown to activate caspase1. Caspase1 activation appears to be dependent on a rod protein in the type III secretion system that contains a flagellin-like motif (52, 53).

Translocation of bacteria through an inflamed gut wall leading to bacteremia sets the stage for further induction of pro-inflammatory cytokines and perturbation of the clotting cascades. As IL-6 is elevated in the CSF of SIDS cases (65), it is reasonable to surmise that pro-inflammatory cytokines might be responsible for observed organ changes: brain microglial response (10); increased brain weight; myocardial acute inflammatory reaction (66). Pro-inflammatory cytokines are responsible for perturbation of the clotting cascade and loss of endothelial integrity (67) resulting in raised fibrin degradation products and the vasculopathy evidenced as intrathoracic petechial hemorrhages. SIDS babies frequently carry a low-producer polymorphism for the anti-inflammatory cytokine IL-10 (68), which could contribute to reduced control of pro-inflammatory cytokine release.

B. thetaiotaomicron

B. thetaiotaomicron, a Gram-negative obligate anaerobe, colonizing the human gut, is a major endosymbiont of the human gut. It can hydrolyze and utilize as an energy source non-digestible polysaccharides and maltooligosaccharides (69). It contributes to bacteria–host symbiosis, postnatal intestinal development, physiology and metabolism of the host (70), and development of the immune system (71).

Bacteroides thetaiotaomicron is recognized as a major player in the adult intestinal microbiome and is useful as a model for the investigation of human–bacterial interactions. It degrades plant polysaccharides. In the infant, it is important during transition from breast milk to a high plant starch diet. It has been shown to stimulate intestinal angiogenesis in response to microbial products reaching the Paneth cells (72). Postnatally, *B. thetaiotaomicron* mediates formation of the mucosal gut barrier assisting in protection against pathogenic invasion seemingly through its effect on expression of species-specific protein antibiotics (73). *B. thetaiotaomicron* allows for adaptive carbohydrate foraging through its environmental sensing system (74). This results in stabilized food webs, and sustenance of bacterial communities (73). This could be important in protection against bacteria and/or toxins purportedly involved in SIDS pathogenesis. The species has been identified among a higher proportion of SIDS infants (30%) compared with control healthy babies (8.8%) (17).

Clostridium spp

Lecithinase-positive clostridia and other clostridia are found significantly more often in formula-fed babies than breast-fed babies (75). Our study found SIDS babies had significantly higher rates of colonization with these anaerobes than live comparison babies (17). Formula feeding seemed to show a trend toward higher colonization rates with clostridia than breastfeeding (17). The role of clostridia in gut inflammation is discussed below. In the context of inflammation, the review by Schuijt et al. (76) considered the hypothesis that the gut has an important detrimental role in promoting systemic inflammation and infection in the critically ill. They demonstrated that during stress and mucosal hypoxia, the mucosa is damaged and host defenses break down allowing bacteria and toxin translocation thought to produce overwhelming inflammation, sepsis, and multiorgan failure (77, 78).

Evidence of Infection in SIDS

Babies dying of SIDS are reported to have sweat-soaked clothing and bedding indicating a febrile episode in the last sleep (61). Rectal temperatures at autopsy of SIDS babies are elevated providing further evidence of fever during the last sleep (79). The basis for a link between the gut microbiome and SIDS is founded upon a substantial body of published evidence and congruence with factors associated with sepsis (**Table 1**). This provides an explanatory scheme for underlying pathogenic mechanisms involved in events leading to SIDS.

Pathological Findings

Consistent pathological findings include intrathoracic petechiae; liquid heart blood; heavy wet lungs; large heavy brain with microglial response (10); acute myocardial inflammatory reaction (66); elevated fibrin degradation products (80); and recent viral infection (61). These suggest a single, common patho-mechanism (5, 6). The finding of *S. aureus* and *E. coli* and other coliforms in normally sterile sites (66, 81, 82) supports the idea of bacteremia occurring as a plausible near-terminal event. Morris' Common Bacterial Toxin Hypothesis (1, 2, 83) first indicated a key role of bacteremia in the final pathway. How the purported bacteremia arises seems to implicate gut integrity/permeability/immunity, which depend on the gut microbiome (84). Potentially, pathogenic *Clostridia* species are over-represented in SIDS babies (17); these could influence the integrity of the gut wall through either induction of an inflammatory response or mechanism involving disruption of EC tight junctions leading to increased bacterial translocation (51).

Inflammation and Pro-inflammatory Cytokines

Inflammasomes are thought to be key in induction of inflammation through production of pro-inflammatory cytokines. Secretion of IL-1β mediates recruitment of cells to the site of proposed clostridia-mediated insult and could play a role in SIDS (52, 53). While gut inflammation is considered to be primary in the events leading to SIDS, the other side of the equation is defective responses to infection, e.g., a defective pathogen recognition pathway.

Clinico-Physiological Events in SIDS

The clinical/physiological events observed in SIDS cases captured on memory monitors (85) can be explained by development of septic/toxic shock resulting from the release of pro-inflammatory cytokines secondary to a bacteremic episode or toxemia. These include fever, tachycardia followed by profound bradycardia, hypoxemia, and gasping (after asystole) (85).

Evidence of Bacteremia/Toxemia in SIDS Infants

The relatively low rate of sterile site infection (16–19% of SIDS cases) could argue against the bacteremia hypothesis. The finding of viable bacteria in a normally sterile site depends on the bacteria's cultivability. Most of the gut microbiota are unlikely to grow on standard diagnostic artificial culture media (86), and this could contribute to low-positive culture rates. It remains highly probable that non-cultivable bacteria entering the blood stream could induce the cytokine storm thought to underlie the final events in SIDS.

Brain and Heart Abnormalities in SIDS

The findings of brain abnormality (87) and of smaller than normal heart and kidneys (88) seem to relate to abnormal prenatal development. This was refuted by Guntheroth and Spiers (89) and needs to be revisited. The smaller than normal heart (with accompanying possible predisposition to arrythmia) could make the infant more vulnerable to profound immuno-inflammatory events associated with bacteremia/sepsis.

SIDS Risk Factors

Risk factors for SIDS can be categorized into genetic, prenatal, and postnatal. These have been described previously (5, 6). Why prone sleep position is a risk factor has been explained by the acquisition of bacteria from contaminated surfaces (sofas, parental bed, being especially contaminated and high risk) (17). It is important to note that data purportedly showing a relationship between bedsharing and SIDS and inferring overlaying/asphyxia as cause (90) needs re-examination because the reason for bedsharing remains poorly described in published studies. It could be interpreted that bedsharing on the night of the last sleep came about because the baby was unsettled or unwell, indicating subtle symptoms of infection. This is supported in a systematic review of bed sharing in which illness and crying were frequently reported (91). The role of alcohol and drugs by co-sleeping parent(s) has also been interpreted as leading to overlaying. The alternative suggestion of lessened awareness in the co-sleeping parent might lead to failure to notice subtle symptoms of illness in the baby. Using overlaying with consequent asphyxiation as the major explanation of the risk factor of bedsharing requires re-evaluation. Mainstream researchers attribute a "respiratory" cause for intrathoracic petechiae. Differentiation between the intrathoracic petechiae observed in asphyxia and SIDS have been well described (92, 93) and show clear differences; yet, these findings are largely ignored by mainstream researchers.

Future Research

Delineation of the gut microbiome in SIDS and healthy babies might provide additional clues to the processes underlying the role of the gut in SUDI. Extended immunohistological examination of the gut wall for evidence of ILCs and subtle gut inflammatory changes in SIDS babies might provide evidence of inflammation contributing to a functional failure of the gut wall to prevent bacterial translocation from the gut lumen into the bloodstream. Nfil3 gene polymorphisms should be sought as these could contribute to gut vulnerability. In the context of bacteremia, efforts using PCR technology should be made to exclude/demonstrate the presence of non-cultivable bacteria in normally sterile sites. The gut microbiome observed in SIDS might correlate with the presence of ILCs and a "pro-inflammatory gut microbiome." Such findings might contribute to a new definition of SIDS. The corollary of establishing a possible "anti-inflammatory" gut microbiome in healthy babies via diet or other means (pre- or probiotic) could be explored as a "natural" mechanism of protecting babies in the future but not forgetting the messages advocating a safe sleeping environment and parental avoidance of cigarette smoke and other harmful drugs.

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The genomic load of deleterious mutations: relevance to death in infancy and childhood

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The human diploid genome has approximately 40,000 functioning conserved genes distributed within 6 billion base pairs of DNA. Most individuals carry a few heterozygous deleterious mutations and this leads to an increased risk of recessive disease in the offspring of cousin unions. Rare recessive disease is more common in the children of cousin marriages than in the general population, even though <1% of marriages in the Western World are between first cousins. But more than 90% of the children of cousin marriages do not have recessive disease and are as healthy as the rest of the population. A mathematical model based on these observations generates simultaneous equations linking the mean number of deleterious mutations in the genome of adults (*M*), the mean number of new deleterious mutations arising in gametogenesis and passed to the next generation (*N*) and the number of genes in the human diploid genome (*L*). The best estimates are that *M* is <7 and *N* is approximately 1. The nature of meiosis indicates that deleterious mutations in zygotes will have a Poisson distribution with a mean of $M+N$. There must be strong selective pressure against zygotes at the upper end of the Poisson distribution otherwise the value of *M* would rise with each generation. It is suggested that this selection is based on synergistic interaction of heterozygous deleterious mutations acting in large complex highly redundant and robust genetic networks. To maintain the value of *M* in single figures over many thousands of generations means that the zygote loss must be of the order of 30%. Most of this loss will occur soon after conception but some will occur later; during fetal development, in infancy and even in childhood. Selection means genetic death and this is caused by disease to which the deleterious mutations predispose. In view of this genome sequencing should be undertaken in all infant deaths in which the cause of death is not ascertained by standard techniques.

Keywords: whole genome sequencing, deleterious mutations, sudden unexpected death in infancy, bacterial toxins, proteomics, molecular autopsy

INTRODUCTION

Infant mortality rates have fallen progressively in UK in the last 50 years, from 18 infant deaths per 1000 live births in 1970 to 4/1000 in 2012 (1). The majority of infant deaths occur in the first month of life (70% in England and Wales in 2012). The post-neonatal mortality rate (infant deaths between 1 and 12 months of age) has also fallen from over 5/1000 live births in 1970 to 1.2/1000 in 2012. The majority of infant deaths after the first month of life are due to sudden unexpected death in infancy (SUDI). The frequency of this condition fell markedly between 1988 and 1995 coinciding with a move from prone to supine sleeping. This followed epidemiological studies, which identified the risk of the prone sleeping position in the early months of life (2, 3). It is one of epidemiology's great triumphs. The number of infant deaths that remain unexplained after a detailed autopsy has, however, leveled off in recent years and there are still approximately 300 such infant deaths (0.5/1000) each year in England and Wales.

Sudden unexpected death in infancy is more common in infants with young single mothers, or mothers with partners who are unemployed (4). The parents commonly smoke and there is an increased incidence of drug abuse. The infants are less likely to have been breast fed and the parents are less likely to follow national guidelines on safe sleeping practice. SUDI is a condition associated with poor social circumstances and if those conditions could be improved the number of cases would undoubtedly fall. Indeed, the number of cases would fall if all that happened was parents stopped smoking. But it is naïve to believe that the condition could be eliminated in this way. Furthermore, emphasis on poor parenting is of no comfort to those mothers whose infants died in spite of exemplary care and there are many such cases.

In my opinion, the infant autopsy in the twenty-first century should be improved. It should include genome sequencing and analysis of body fluids for bacterial secretory products as well as the standard dissection and histological examination of tissue. This is the molecular autopsy to supplement the standard autopsy. The current methods for assessing infection are inadequate and there is plenty of evidence to indicate that analysis of the genome will provide valuable information in many cases (5). Parents have a right to know why their infant died, and pathologists have a duty to do all that they can to answer that question. Lecturing parents

on their inadequacy is no substitute for a full scientific assessment of the cause of death.

In this article, I make a case for the use of whole genome or whole exome sequencing in the infant autopsy. Initially, it will be used for cases where death is unascertained after a detailed standard autopsy. It should also be used in cases of childhood death when the cause is unclear. But eventually, it will be used in all infant deaths because the information supplied will be of considerable value in the broader understanding of the function of human genes as well as for genetic counseling. Genome sequencing should be mandatory in cases of infant death attributed to the shaken baby syndrome, because the evidence for trauma is often weak and some of these cases could be due to natural disease (6). Avoiding a single prosecution will save many multiples of the $2000 estimated cost of whole genome sequencing.

NEUTRAL MUTATIONS

If two homologous strands of DNA from an autosomal chromosome from the same person or from a different person are compared over 99% of the bases will be exactly the same (7). The same will apply to the X chromosome and to the Y chromosome when they are compared between individuals. However, discrepancies do occur in approximately 1 in 300 bases and these base changes are due to mutations in previous generations. The rate of mutation when DNA is copied in mitosis is of the order of 5×10^{-10} bp per mitotic division (8). The number of bases in the diploid genome is 6×10^9; thus, approximately three bases will be miscopied each time a cell divides. There are over 40 divisions between the zygote and the oocyte, and over 60 between the zygote and spermatozoa. Thus, each individual will have over 100 base changes due to mutations occurring in parental gametogenesis. These are private mutations, they are unlikely to be present in any other member of the human race and they are not shared with siblings.

An individual will also inherit over 100 mutations that arose during grand-parental gametogenesis. These are also private mutations, but they will be present in the genome of parents and will be shared with siblings. Another 100 mutations will have arisen in the gametogenesis of great grandparents, and a further 100 from mutagenesis in great great grandparents, and so on back through thousands of generations. Obviously, grandparents produce twice as many mutations as parents because there are four grandparents and only two parents. But only half of the mutations are passed on in each generation so the number of mutations acquired per individual remains the same at over 100 for each generation.

If the current human variation is 1 in 300 bases and 100 new mutations arise per generation then the process has been going on for 100,000 generations or approximately 2.5 million years. Only 1–4% of the human genome is conserved; this is the protein coding and regulatory part of the genome (7). Thus, the majority of base changes are neutral in evolutionary terms and there is no selection against these mutations. But let us now consider the fate of a new neutral mutation entering the genome of an individual. If that individual has two children then there is a 25% chance that the mutation is not present in the genome of either child; a 25% chance that it is present in the genome of both children and a 50% chance that one child has a copy and the other does not. A similar stochastic process occurs in subsequent generations and the result is that most new neutral mutations are lost purely by chance but a few increase in number from generation to generation, again purely by chance. Fisher was able to show that in a stable population the chance of a new neutral mutation surviving for n generations is $2/n$, when n is large. But the number of copies, if it does survive, will be close to $n/2$.

Those neutral mutations that survive will gradually expand in the population over many generations and some will eventually be present in over 1% of the human race. These are the common single nucleotide polymorphisms used in genome wide association studies (GWAS). The majority of these have been in the human genome for over 10,000 generations.

Mutations in protein coding genes and regulatory genes can also be neutral. A base change in a protein coding gene that does not alter the amino acid sequence of the protein is termed a synonymous change. These are commonly neutral. A base change that alters the amino acid sequence is likely to be deleterious but can be neutral. In the latter situation, the new protein is different but works as well as the wild type protein. Or it could be that the new protein is advantageous in certain situations and disadvantageous in others; but neutral overall. Equally, regulatory changes can cause faster or slower and stronger or weaker responses. This, once again, might be advantageous in certain situations and disadvantageous in others. These neutral changes will have similar kinetics to the neutral base changes in non-coding regions described above. The main genetic differences between individuals and between races are due to neutral mutations in protein coding and regulatory genes.

There is some evidence that in certain situations the heterozygote can have an advantage over the homozygote for a particular locus (9). This applies in particular to the HLA system of genes. These loci are highly polymorphic and it appears that the heterozygote is better at dealing with infection and avoiding autoimmune disease than the homozygote. Indeed, bacteria and viruses adapt by evolution to their hosts and new neutral mutations can thwart that adaptation, at least for a few generations. Bacteria adhere to surface proteins on cells and the heterozygote will have fewer proteins of any one type than the homozygote; this again could be advantageous. In certain infections, a strong regulatory response is required, while in others a weaker response might be advantageous. The individual with both responses in their repertoire will be at an advantage.

DELETERIOUS MUTATIONS

A deleterious mutation is one in which the protein product of a gene is not produced, is produced, and does not function, or is produced and interferes with normal function. Equally, mutations in regulatory elements can be deleterious if the regulatory function is impaired. These mutations can arise from single base changes or more extensive insertions, deletions, or frame shifts. The majority of base changes due to mutation in spermatogenesis and oogenesis lead to neutral mutations in the genome and the best available estimates are that the mean number of new deleterious mutations arising in each generation is between 0.5 and 1.5 (10).

Let us define M as the mean number of deleterious mutations in the germ line of adults and N as the mean number of

new deleterious mutations arising in gametogenesis and passed on to the next generation. Thus, the mean number of deleterious mutations in zygotes is $M+N$.

If first cousins marry, they have an increased risk of bearing children with recessive disease. This reflects the presence of heterozygous deleterious mutations in their grandparents that have been passed to both cousins and then appear in the homozygous form in their children. This indicates that we all carry a few deleterious mutations and the value of M is >1. But over 90% of the children of cousin marriages do not suffer from a recognizable recessive disease and are as healthy as the rest of the population. This indicates that M is a small number and is probably <10 (10).

The nature of meiosis indicates that deleterious mutations will be distributed at random during the formation of spermatozoa and oocytes. Thus, there will be a Poisson distribution of deleterious mutations in zygotes (**Figure 1**) with a mean <10. A more precise model of this process has been published and the best estimates obtained were $M=4$–8 and $N=0.5$–1.5. These estimates were based on the assumption that there are 30,000 genes in the haploid set (10). The current estimate is closer to 20,000 and this means the value of M will be proportionally smaller.

If one new deleterious mutation enters the genome per generation and the mean number of deleterious mutations in adults, after many thousands of generations, is still in single figures, then there must be strong selection against the deleterious mutations. This will operate at the upper end of the Poisson distribution. The zygotes that carry the most deleterious mutations will be the least likely to survive and develop into infants and eventually adults.

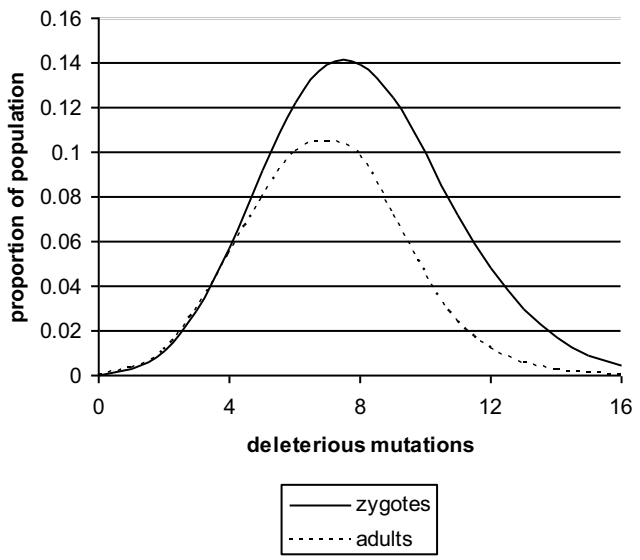

FIGURE 1 | The distribution of deleterious mutations in zygotes and in adults, based on a previously published theoretical model (13). The deleterious mutations interact synergistically to impair the performance of complex genetic systems. Zygote loss will be mainly immediately post conception. The mean number of deleterious mutations (M) in UK at present is probably less than shown in the figure. It would fall even further if N fell due to improved social conditions.

The number of zygotes lost by this process is not inconsiderable. The mean in zygotes is $M+N$, and the mean in adults is M. To achieve this change, approximately 30% of the zygotes at the upper end of the Poisson distribution will fail to develop (11–13).

The process envisaged, and previously described, is that heterozygous deleterious mutations interact synergistically to impair the performance of large complex genetic systems during development. The individual systems consist of hundreds or thousands of genes. A model along the following lines can explain what is observed (10–13).

A. Zygotes, which have four deleterious mutations in an essential genetic system, will not survive. Most of the loss will occur shortly after conception, but a few deaths might arise later in pregnancy or even in early infant life.
B. Zygotes, which have three deleterious mutations in an essential system, are likely to survive and be born alive. The system will function adequately most of the time, but will be less robust than normal, and will be at risk of malfunction in response to environmental stress. These infants might die in infancy or childhood due to infection.
C. Zygotes with two deleterious mutations in an essential system are likely to survive. Their system will function adequately most of the time and will only be seriously compromised by a major environmental stress. These infants could also die in infancy due to infection.
D. Zygotes with one deleterious mutation in an essential system will survive and their system will be robust and will function adequately. They are unlikely to die in infancy.
E. Zygotes with zero deleterious mutations have systems, which work beautifully. They grow to be intelligent and healthy adults and have all the luck.

GENDER DIFFERENCES

Males are more likely to die than females in every year from birth to old age (9, 14). They are also more likely to die *in utero*. In SUDI, there is a constant ratio of three male deaths for every two female deaths (15). The main genetic difference between males and females is that males have only one X chromosome, while females have two. The X chromosome carries approximately 5% of the genome and thus carries in the region of 1000 genes (7). Every male is 1000 genes short of a full set – it explains a great deal.

Female cells, however, express the same number of genes as male cells. This is because one of the X chromosomes in every female cell is inactivated. This is a random process occurring in stem cells. It means that every female is actually two slightly different genetic individuals within one body – this also explains a lot.

Inbreeding depression is an intriguing phenomenon observed in laboratory animals (16, 17). Brother sister mating through many generations leads to genetic homogeneity. Heterogenous changes are gradually lost and each genetic locus contains identical genetic material. These animals, however, are sickly. There is impaired development *in utero*, increased rates of fetal death, low-birth weight, increased infections in early life, and a shortened life-span. There are two possible mechanisms that explain inbreeding depression, one is recessive disease and the other is loss of heterozygous advantage.

Table 1 | This table shows the relative risk of SUDI in males with a deleterious mutation on X, assuming that the excess is caused by sex linked recessive disease.

Relative risk of SUDI in males with a deleterious mutation on the X chromosome	% of males with a deleterious mutation on the X chromosome	
	SUDI (%)	CONTROLS (%)
3	50	25
6	40	10
11	37	5

If 25% of control males have a deleterious mutation on X then 50% of SUDI males will carry a deleterious mutation on X and the relative risk of SUDI will rise threefold. Relative risk is the risk of a male with a deleterious mutation on X compared with a male without a deleterious mutation on X. If only 5% of control males have a significant deleterious mutation on X then 37% of SUDI males will have an X-linked mutation and the relative risk will rise 11-fold. The calculations are shown in the Section "Appendix."

1. Recessive disease: there is a selection against deleterious mutations during the process of producing genetic homogeneity and therefore inbred animals have a reduced number of deleterious mutations. However, any deleterious mutations that survive will be homozygous and therefore cause recessive disease.
2. Loss of heterozygous advantage: heterozygous loci confer advantage in fighting infection as discussed above in the section on neutral mutations.

The relevance of inbreeding depression to male death in infancy is that males have the equivalent of inbreeding depression on 5% of the genome. A deleterious mutation on X will cause a recessive disease in males but not in females. In females, only 50% of the cells will express the deleterious gene product. Females will still have heterozygous advantage on X as they are capable of expressing both genes, albeit in different cells.

The number of deleterious mutations on the X chromosome will be approximately $M \div 40$, because the X chromosome has 5% of the genome and we are considering the diploid cell. In fact, the number is probably less in males because some deleterious mutations on X will be lethal in the male. The figures in (**Table 1**) are calculated by assuming that the increased male deaths in SUDI are entirely due to X-linked deleterious mutations. The increased risk of SUDI with a deleterious mutation on X will be between 3- and 11-fold, if the assumption is correct. This indicates the potential value of sequencing the X chromosome in SUDI.

If X-linked recessive disease is not the explanation, or the sole explanation, then the alternative is loss of heterozygous advantage. This would point to infection as a likely cause and indicates that the molecular autopsy must have two arms: genomics and proteomics.

GENOME WIDE ASSOCIATION STUDIES IN INFANT DEATH

In GWAS, up to 500,000 common polymorphisms are assessed in a large cohort of individuals with a specific disease and this is compared with a large cohort of controls from the general population (18–32). Common polymorphisms are those that are present in over 1% of the population. These are neutral changes that have been in the population, without effective selection, for many thousands of generations. Most of the polymorphisms occur in the non-coding regions of the genome but they will be linked to regulatory elements and conserved functioning essential genes. The impetus for these studies was the concept that common mutations cause common disease and the expectation was that there would be strong association between a small number of polymorphisms and the disease under investigation. This has not been borne out in practice. In schizophrenia, manic-depressive psychosis, multiple sclerosis, hypertension, and type 2 diabetes mellitus a large number of polymorphisms are found to be weakly associated with the disease (18–32). The high heritability of schizophrenia and manic-depressive psychosis, for example, is not explained by neutral polymorphisms (28, 29). These results should have been anticipated, because neutral mutations are neutral in evolutionary terms and are not the main genetic cause of disease. Disease is a consequence of deleterious mutations, against which selection operates.

In the GWAS undertaken so far, most of the associations between a polymorphism and a disease have odds ratios <1.5. The significance level used in these studies is set at 0.05 for each polymorphism but since these assays test many hundreds of SNPs there needs to be a correction for multiple testing such that the reporting of false positives (or negatives) is minimized. In view of this large cohorts are required to establish statistical significance and positive associations should be independently checked in a replication set. However, if the odds ratio is raised then this does give a clue to causation. Because it indicates that a linked gene influences the risk of disease. This is particularly relevant if the linked genes have a role in infection or inflammation. Neutral mutations are not the main cause of disease but they can influence the risk of disease by small margins. A neutral mutation can be disadvantageous in relation to one organism but with a compensatory advantage in relation to another; neutral overall in evolutionary terms.

In general, in epidemiological studies, association does not equal causation. But there is an interesting argument that in GWAS association does equal causation. This is because in meiosis there is perfect randomization of neutral polymorphisms and this eliminates confounding factors. The perfect randomized trial. Not everybody agrees with this idea, particularly since it depends on there being no bias in the selection of the control population. However, it does appear that associations, once established, give clues to causation.

I am aware of one GWAS of a cohort of German SIDS cases. This study was funded by the Foundation for the Study of Infant Death (FSID), which has now been renamed the Lullaby Trust. The results have been presented at a number of international meetings but not yet published in full. This study found a number of potential associations with odds ratios between 1 and 1.5. One of the associations reached statistical significance; the odds ratio was 1.5 and the upper bound of the 95% confidence interval was <2. The research workers concerned are planning to enlarge the cohort prior to publication. There are no details at present about the specific loci (33).

Genome wide association studies can also provide information on copy number variants in the experimental and control populations. The larger copy number variants are more likely to indicate deleterious mutations and this can provide useful additional information (34).

There have been a number of studies of cytokine regulatory genes in SIDS (35–40), which are reviewed in detail in an accompanying article (see Ferrante and Opdal) These studies are necessarily smaller than GWAS and do not have the large control cohorts for comparison. There is, however, some evidence that the balance of pro- and anti-inflammatory cytokine responses can influence the response to infection in general and influence the risk of SIDS. Interaction between smoking and the cytokine response is a particular interesting area of investigation. Time will tell whether or not GWAS confirms that cytokine regulatory genes have a role in SIDS.

GENOME SEQUENCING IN INFANT DEATH

It is now possible to sequence the genome at a cost, which is comparable to that of the standard infant autopsy. Whole genome screening or whole exome screening can be undertaken for around $2000 and the price is still falling. This is the investigation, which will reveal the extent to which genetic factors predispose to infant death. The interpretation of the findings, however, will still be extremely difficult, at least initially. Recognizing the small number of deleterious mutations in highly conserved essential genes and distinguishing these from the vast number of other changes in the genome will not be easy, but it is a tractable problem.

In SUDI, the following findings are anticipated:

1. In some cases, a single deleterious mutation in an essential gene will be, in itself, a sufficient explanation for death. We already know that mutations in cardiac channelopathy genes are responsible for approximately 10% of SUDI cases (41). This information has come from sequencing only seven genes. If the entire genome is sequenced it is inevitable that further examples will be found. Some of the mutations will arise *de novo* and some will be present in the germ line of parents. Even in these cases, synergistic interaction with other deleterious mutations might be important and there will be some environmental trigger, such as infection. Recognizing these single deleterious mutations and working out, which are *de novo* will be important for genetic counseling of the families.
2. Single deleterious mutations on X might be a major risk factor for SUDI in males. The male excess in SUDI could be due to sex linked recessives or to a loss of heterozygous advantage as argued above. Sequencing the X chromosome in males is likely to yield a considerable amount of information relevant to infant death.
3. Discerning the role of synergistic interaction of heterozygous deleterious mutations in complex genetic systems will be much more problematical. But it should be possible to determine the actual genetic load of deleterious mutations and relate this to risk of death. Infants with the most deleterious mutations will be at increased risk of death of all types, including SUDI.

POPULATION MONITORING

The genomic load of deleterious mutations is a major factor in health and disease. These mutations contribute to death in infancy and in childhood. This is an inevitable conclusion of the observation that there is strong selection against these mutations. Selection means genetic death caused by disease to which the mutations predispose.

The rate at which new deleterious mutations enter the genome is a random variable, and many factors will influence the rate. Parental age, smoking, diet, and infection are likely to be involved as is environmental pollution. The models used in this paper indicate that if N falls then M will also fall over several generations and the population will become healthier. The rate of infant death will fall. There is a public health imperative to measure and monitor the rate at which new deleterious mutations enter the genome so as to recognize causative factors and avoid them as far as possible.

The considerable fall in the rate of infant mortality and overall improvement in health over the last half century will be due to many factors; but it is highly likely that part of this change is a consequence of a fall in rates of somatic mutation in stem cells, including germ cells. Improved social and economic conditions could bring about this change in a number of ways including better diet, less pollution, and less infection. We cannot, however, assume that this process will continue its beneficial course. Economic progress can lead to more pollution not less, and climate change could have many detrimental effects.

DISCUSSION

In probing the pathogenesis of disease, I follow a simple but powerful maxim: germs cause disease, genes act in complex networks to prevent disease. In so far, as this idea is correct, and it will only be correct in part, we can anticipate that common disease will be due to common organisms. Our attention should therefore focus on bacteria of the normal microbial flora and how they could interact with a genome impaired by deleterious mutations (42, 43).

Whole genome sequencing and whole exome sequencing are new techniques, which were not available in the twentieth century. These techniques should allow us to define precisely the contribution of genetic mutation to infant death. But interpretation of the results of the analysis will not be easy and it will be some time before we are confident in recognizing the significant changes. The cost of genome sequencing is not inconsiderable, but it is comparable with the total current cost of an autopsy. In cases were the cause of infant death is a matter of legal dispute and criminal charges are considered genome sequencing could lead to considerable cost saving (6).

Disease is an interaction between environmental stress and impaired genetic systems. Thus, genome sequencing will not provide a complete answer. It should be supplemented with the other arm of the molecular autopsy, which is proteomic analysis of body fluids. In particular, we need to seek and identify bacterial secretory products in body fluids in order to diagnose infection (5). This article, however, concentrates on the genetic aspects because the genetic techniques are now ready for direct application; the proteomic techniques are still in the phase of development.

A common counter-argument to the ideas presented in this article is that nothing can be done about our genetic constitution and sequencing the genome will not lead to any preventive strategies. In my view, this is a misconception. Deleterious mutations are ultimately caused by environmental factors and if we can identify the factors then the rate of mutation can be decreased. Indeed, falling rates of somatic and germ line mutation are probably a major factor in the improvement in health we have seen over the last 50 years in the technologically advanced countries. Smoking is mutagenic and if we could measure this directly it would be a powerful incentive for young parents, contemplating a family, to quit the habit.

In my experience, the question that parents want answering is "why did my infant die?"; and this takes precedence in their mind over discussions related to preventive strategies. The job of the pathologists is to answer that question. We were unable to do so in many cases in the twentieth century because the techniques of the molecular autopsy were not available. Genome sequencing is now available and should be used. Proteomic techniques will follow in the near future.

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APPENDIX

The calculations are based on the assumption that the excess male death in SUDI is entirely due to heterozygous deleterious mutations on X.

Consider the case in which 25% of males have a deleterious mutation on X.
In 100 cases of SUDI, there will be 60 males and 40 females.
The 20 excess males all have a deleterious mutation on X.
About 25% of the remaining 40 males also have a deleterious mutation on X.
Thus, 30 of 60 males (50%) have a deleterious mutation on X.
About 25% of the male population produces 50% of the deaths.
About 75% of the population produces the other 50% of the deaths.
Relative risk is $(50 \div 25) \div (50 \div 75) = 3$.

Now consider the case in which 10% of males have a deleterious mutation on X.
In 100 cases of SUDI, there will be 60 males and 40 females.
The 20 excess males all have a deleterious mutation on X.
Just 10% of the remaining 40 males have a deleterious mutation on X.
Thus, 24 of 60 males (40%) have a deleterious mutation on X.
About 10% of the male population produces 40% of the male deaths.
The remaining 90% of the male population produces 60% of the male deaths.
Relative risk is $(40 \div 10) \div (60 \div 90) = 6$.

Finally, consider the case in which 5% of the males have a deleterious mutation on X.
Twenty-two males of 60 (37%) SUDI deaths will have a deleterious mutation on X.
Thus, 5% of the population of males produces 37% of the deaths.
The remaining 95% of the population produces 63% of the deaths.
Relative risk is $(37 \div 5) \div (63 \div 95) = 11$.

Please note that the relative risk for specific deleterious mutations will vary markedly. The calculations relate to the mean of the distribution. The mean is likely to be a constant regardless of time, population and race; hence the remarkable constancy of Mage's ratio – three male deaths for every two female deaths (15).

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Sudden infant death syndrome and the genetics of inflammation

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Several studies report signs of slight infection prior to death in cases of sudden infant death syndrome (SIDS). Based on this, a hypothesis of an altered immunological homeostasis has been postulated. The cytokines are important cellular mediators that are crucial for infant health by regulating cell activity during the inflammatory process. The pro-inflammatory cytokines favor inflammation; the most important of these are IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-18, TNF-α, and IFN-γ. These cytokines are controlled by the anti-inflammatory cytokines. This is accomplished by reducing the pro-inflammatory cytokine production, and thus counteracts their biological effect. The major anti-inflammatory cytokines are interleukin-1 receptor antagonist (IL-1ra), IL-4, IL-10, IL-11, and IL-13. The last decade there has been focused on genetic studies within genes that are important for the immune system, for SIDS with a special interest of the genes encoding the cytokines. This is because the cytokine genes are considered to be the genes most likely to explain the vulnerability to infection, and several studies have investigated these genes in an attempt to uncover associations between SIDS and different genetic variants. So far, the genes encoding IL-1, IL-6, IL-10, and TNF-α are the most investigated within SIDS research, and several studies indicate associations between specific variants of these genes and SIDS. Taken together, this may indicate that in at least a subset of SIDS predisposing genetic variants of the immune genes are involved. However, the immune system and the cytokine network are complex, and more studies are needed in order to better understand the interplay between different genetic variations and how this may contribute to an unfavorable immunological response.

Keywords: genetics, immune system, interleukins, infection, SIDS

INTRODUCTION

Sudden infant death syndrome (SIDS) is defined as the sudden unexpected death of an infant <1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history (1). Even though there have been numerous studies trying to understand the pathophysiological mechanisms of SIDS, we still not fully understand what causes these deaths, or how to prevent them. The fatal triangle developed by Rognum et al. (2) suggest that SIDS occur when an infant at the same time is in a vulnerable developmental stage, with a rapid development of both the central nervous system (CNS) and the immune system, has predisposing factors such as an unfortunate genetic "make-up" or brainstem astrogliosis, and that trigger events such as slight infection, prone sleeping, maternal smoking, or overheating are present (**Figure 1**) (2).

SIDS AND INFECTION

There is compelling evidence for a dysfunctional immune response in SIDS, and already in 1947, Werne et al. suggested that respiratory infection was the cause of death in an otherwise healthy infant (3). Since then, there have been numerous reports and papers describing signs of slight infection in SIDS infants (4–7). An immunological "overreaction" has been postulated since about half of the SIDS victims have had symptoms of slight infection in the days before death (8, 9).

Arnon et al. have hypothesized that some infants might suffer respiratory arrest due to botulinum toxin produced by *Clostridium botulinum* (10). From a cohort of 280 infants, they showed that botulinum toxin was present in 10 infants, of which 9 had been diagnosed as SIDS (10). Stoltenberg et al. have reported immune stimulation in both the upper airways and intestines, showing that SIDS had higher number of IgM immunocytes in the tracheal wall than controls, but significantly lower numbers of IgA and IgM immunocytes than cases of infectious death (11). In the duodenal mucosa, the number of IgA immunocytes was higher in SIDS cases than in controls. These findings indicate that the mucosal immune system is activated in a large proportion of SIDS (11). It is also shown that SIDS has higher IgG and IgA immunocyte density in the palatine tonsillar compartments than controls (12). Furthermore, salivary glands have a higher number of CD45+ stromal leukocytes, as well as intensified epithelial expression of HLA-DR and secretory component, and increased endothelial expression of HLA class I and II (13). These observations confirm that the immune system is activated in SIDS, probably with release of certain cytokines that are known to up-regulate epithelial expression of HLA-DR and secretory component (13).

A real breakthrough for the immunological overreaction theory was the demonstration by Vege et al. (8, 14), who showed that SIDS victims who have had signs of slight infection prior to death had both increased number of IgA immunocytes and HLA-DR expression in their laryngeal mucosa, as well as increased levels of IL-6 in their cerebrospinal fluid (CSF). In fact, half of the SIDS victims had CSF IL-6 concentrations in the same range as victims of meningitis and septicemia (8). A further support for the infection theory is a study performed on registry data from Norway and Sweden, which suggests that there is a co-variation between epidemics of whooping cough and SIDS (15). The association was stronger in Sweden than in Norway, which may reflect that Swedish infants are not vaccinated against *Bordetella pertussis* while the Norwegians infants are (15).

Stray-Pedersen et al. showed that SIDS victims with positive *Helicobacter pylori* stool antigen (HpSA) immunoassay had elevated IL-6 in the CSF compared to SIDS victims with negative HpSA test (16). Furthermore, detection of *Helicobacter pylori* antigen in stool was found associated with SIDS and death due to infection, indicating that this bacteria may represent a contributing factor to sudden death during the first months of life (16).

Surfactant protein A (SP-A) is a protein produced in the lungs, with a major purpose to reduce the surface tension at the alveolar air–liquid interface. Furthermore, it takes part in regulation of the inflammatory process. Interestingly, with regard to SIDS, there is a drop in alveolar SP-A expression in the first months after birth (17), corresponding to the classical age peak of SIDS. Thus, it may be hypothesized that this transient low expression of SP-A may be a part of the increased vulnerability for SIDS at that age (17).

It is also suggested that *Staphylococcus aureus* (*S. aureus*) are involved in events leading to SIDS (18). Based on observations from samples collected from the intestinal tract in SIDS compared with samples from feces from a group of healthy controls, it was shown that *S. aureus* and staphylococcal enterotoxins were more prevalent in SIDS. However, as much as 40% of the controls were positive for *S. aureus*, indicating that this bacteria is common in infants, and that the detection may not be seen as a support for the diagnosis of SIDS (18).

Another study investigated pyrogenic toxins of *S. aureus* in SIDS infants from different countries (19). The study reported these pyrogenic toxins in >50% of SIDS infants from three different countries; Scotland, France, and Australia, and suggest that further investigation into the effect of the toxins may be important (19). A study by Blackwell et al. found that the prevalence of *S. aureus* in nasopharyngeal flora was significantly higher in SIDS cases compared to age-matched healthy controls (20). Furthermore, SIDS found in a prone sleeping position more often had symptoms of slight infection prior to death than the babies put to sleep on their back.

The relationship between laryngeal immune stimulation, clinical signs of slight infection prior to death, and high levels of IL-6 in CSF may indicate an interaction between the immune system and the CNS (14). The assumption of such a relationship is strengthened by the recently reported increased IL-6 receptor expression on serotonergic cells in brain stem nuclei involved in respiratory regulation in SIDS cases compared to controls (21). Almost half of the investigated SIDS cases had signs of mild infection prior to death, and the study provides evidence for aberrant interactions in SIDS infants between IL-6 and the area of the brain stem involved in protective responses to hypercapnia, potentially induced by the combined effect of prone position and mild infection.

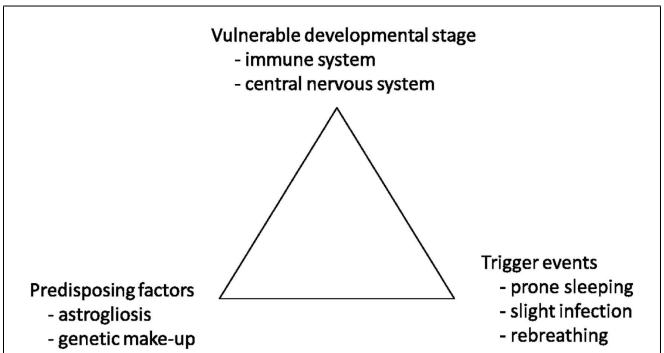

FIGURE 1 | The hypothesis of a fatal triangle in SIDS. Modified with permission from Rognum and Saugstad (2).

COMPLEMENT COMPONENT C4

The first gene involved in the immune system to be investigated with regard to SIDS was the gene encoding complement component C4. This gene consists of two loci, C4A and C4B, and is highly polymorphic. Partial deletions of the C4 gene are common and found in 5–20% of Caucasians (22). The C4 gene has been investigated in both German and Norwegian SIDS victims, but none of these studies detected any differences between SIDS cases and controls with regard to allele frequencies (23–25). However, two of the studies revealed an association between slight infections prior to death and partial deletions of either the C4A or the C4B gene, which may indicate that this combination represents increased risk of sudden infant death (**Table 1**) (23, 25).

Table 1 | C4 and HLA-DR gene variants investigated in cases of SIDS.

Gene	Variant	SIDS[a]	Controls[a]	Findings, reference
C4	Deletion of gene	40	47	In SIDS: association between slight infection prior to death and partial C4 deletions ($p = 0.013$) (23)
	Deletion of gene	39	183	No significant findings (24)
	Deletion of gene	104	84	In SIDS: association between slight infection prior to death and partial C4 deletions ($p = 0.039$) (25)
HLA-DR	Allele analyses	40	47	No significant findings (23)
		39	183	HLA-DR2 associated with SIDS ($p = 0.002$) (24)
	Allele analyses	16	181	Underpowered, no significant findings (26)

[a] *Number of cases.*

HLA-DR

Numerous diseases are associated with different alleles of the major histocompatibility complex, and HLA-DR has been investigated in a few SIDS victims (**Table 1**). There has been reported a significant decreased frequency of HLA-DR2 in a study including 39 SIDS cases and 183 controls (24). However, Schneider et al. (23) investigated 40 SIDS cases and found no significant difference in the HLA-DR gene frequencies between the SIDS cases and the controls, an observation, which was later confirmed in a study of 16 Norwegian SIDS cases (26).

IL-10

The genes most likely to explain the vulnerability to infection seen in SIDS are the cytokine genes. Several studies have investigated these genes in an attempt to uncover associations between SIDS and different genetic variants, and one of the most investigated is IL-10.

IL-10 is an important immune regulatory cytokine that down-regulates the production of pro-inflammatory cytokines, such as IL-6 and TNF-α. Levels of IL-10 control the balance between inflammatory and humoral responses, and IL-10 therefore plays an important role in the development of infectious disease. Variability in IL-10 production has a hereditary component of approximately 75%, and the SNPs in the promoter region in position −1082, −819, and −592, as well as the microsatellite IL-10R and IL-10G, are collectively responsible for the production of the protein (27, 28).

The first study investigating the IL-10 gene in SIDS was by Summers et al., who investigated 23 SIDS cases and found that SIDS was associated with both the ATA haplotype and the −592A allele (29). However, according to the authors, babies who died of other causes might have been included in the SIDS group, which may have influenced the results (29). A Norwegian study of 214 SIDS cases was unable to confirm the association to SIDS, but found an association between the ATA haplotype and the ATA/ATA genotype and infectious death (**Table 2**) (30). The latter study also investigated the microsatellites, and found a higher percentage of the genotypes G21/G22 and G21/G23 in cases of infectious death compared to SIDS, and a higher percentage of G21/G22 in the SIDS cases compared to controls, while there were no differences between the groups for the IL-10R area (**Table 2**) (30). Subsequent studies investigating this gene have reported conflicting results, a small study investigating that 38 SIDS cases found an association between −592A and ATA/ATA and SIDS (31), while other studies did not (**Table 2**) (32, 33). An Australian study investigated the −1082A/G polymorphism in SIDS cases and SIDS parents from different countries, but did not find any differences in genotype frequency between this combined SIDS population and controls, neither between SIDS parents and controls (**Table 2**) (34). This study also investigated the influence of genotype and smoking on IL-10 responses. They found that the pooled data from smokers had significantly lower levels of IL-10 responses to TSTT, but there were no significant differences for smokers compared with non-smokers for the three genotypes (34). The lowest levels of IL-10 responses were observed among smokers who were homozygous for the −1082A allele, which is most prevalent among Aboriginal Australians and Bangladeshis. This is interesting, as the major difference between the risk factors for SIDS in these two groups is the level of exposure of infants to maternal smoking.

Based on the findings in these studies, one might speculate that in some situations an infant with an unfavorable IL-10 genotype may exhibit aberrant IL-10 production, which in turn leads to a disturbed immunological homeostasis and an increased risk for sudden death. This may be especially unfavorable if exposed to smoking, in particular *in utero* or if a smoking mother is breast-feeding.

IL-6

IL-6 is an acute phase protein that induces B- and T-cell growth and differentiation. IL-6 is also an important mediator of fever, and influences the effect of other cytokines. The first study of the IL-6 gene in cases of SIDS was a British study that included common polymorphisms in the genes encoding IL-4, IL-6, IFN-γ, TGF, and VEGF (35). They found significant differences for the genes encoding IL-6 and VEGF: the genotypes IL-6 −174GG, and VEGF −1154AA were more frequent in SIDS cases than in controls (**Table 2**) (35). Even though only a small number of SIDS cases were included, the authors suggest that the causation of SIDS is related to both fetal lung development and an infant's innate ability to mount an inflammatory response to infection (35). The findings in the IL-6 gene have been confirmed in a study of Australian SIDS cases (36), but not in a Norwegian study (**Table 2**) (37). In the Australian study, it was in addition found a relationship between IL-6 responses to endotoxin, IL-6 genotype, and smoking status (36). A study by Ferrante et al., investigated the −572G/C polymorphism in the IL-6 gene in 148 SIDS cases, but it did not find any association between this SNP and SIDS (**Table 2**) (38). A study evaluating the correlation between HLA-DR expression in laryngeal mucosa and interleukin gene variation found that 12 of 13 SIDS cases (92%) with high HLA-DR expression, prone sleeping position, and sighs of infection prior to death had the IL-6 −176 CG/CC genotypes ($p = 0.01$) (45).

IL-1

IL-1 is a pro-inflammatory cytokine that induces the synthesis of acute phase proteins, and also induces fever. There are two structurally distinct forms of IL-1: IL-1α, which is the acidic form and IL-1β, which is the neutral form. IL-1 is regulated by the competitive antagonist IL-1Ra. The polymorphisms IL-1β −511C/T and IL-1Ra +2018T/C have a significant effect on the IL-1β levels. An Australian study investigated a combined SIDS group with cases from different countries with European controls, but did not find any association between those SNPs and SIDS (**Table 2**) (40). It was, however, shown that smoking had a significant effect on both IL-1β and IL-1ra responses to endotoxin, and that this effect differed according to genotype (40). This finding is highly interesting, since maternal smoking is one of the most well-known risk factors for SIDS (9, 46, 47).

A Norwegian study investigated a variable number of tandem repeat (VNTR) in intron 6 and the SNP in +4845G/T in the IL-1α gene, as well as the −511C/T polymorphism in the gene encoding IL-1β and a VNTR in intron 2 of the gene encoding IL-1ra (**Tables 2** and **3**) (39). When investigating each polymorphism separately, no association to SIDS was found. However, when combining VNTR

Table 2 | Interleukin and cytokine gene polymorphisms investigated in cases of SIDS.

Gene	Variant	SIDS[a]	Controls[a]	Findings, reference
IL-10	−1082A/G −819C/T −592A/C	214	136	ATA haplotype associated with infectious death ($p = 0.007$), no association to SIDS (30)
	−1082A/G −819C/T −592A/C	38	330	−592A allele associated with SIDS ($p = 0.004$) (31)
	−1082A/G −819C/T −592A/C	23	100	Underpowered, no significant findings (32)
	−1082A/G −592A/C	123	406	No significant findings (33)
	−1082A/G	85	118	No significant findings (34)
	IL-10G	214	136	G21/G22 associated with SIDS ($p = 0.005$) (30)
	IL-10R	214	136	No significant findings (30)
IL-6	−174G/C	25	136	−174G associated with SIDS ($p = 0.034$) (35)
	−174G/C	96	467	−174GG associated with Australian SIDS ($p = 0.02$) (36)
	−174G/C	175	71	No significant findings (37)
	−572G/C	148	131	No significant findings (38)
IL-1α	+4845G/T	204	131	No significant findings (39)
	VNTR intron 6	204	131	VNTR A1A1/+4845TT combination associated with SIDS ($p < 0.01$) (39)
IL-1Ra	+2018T/C	87	122	No significant findings (40)
	VNTR intron 2	204	131	No significant findings (39)
	VNTR intron 2	113	218	2/2 genotype and A2 allele more common in SIDS ($p = 0.026$ and $p = 0.004$, respectively) (41)
	VNTR intron 2	49	94	A2 allele associated with SIDS ($p = 0.007$) (42)
TNF-α	−308A/G	23	330	Underpowered, no significant findings (29)
	−238 G/A −308G/A	23	100	Underpowered, no significant findings (32)
	−1031T/C −857C/T −308G/A −244G/A −238G/A	148	131	−238GG associated with SIDS ($p = 0.022$). SNP profiles −1031CT/−238GG/−857CC/−308GG and −1031TT/−238GG/−857CC/−308AA associated with SIDS ($p = 0.03$ and $p = 0.05$, respectively) (43)
	−308A/G	89	267	−308AA associated with Australian SIDS ($p = 0.03$) (44)
IL-8	−251A/T −781C/T	148	131	IL-8 −251AA/AT and IL-8 −781CT/TT more frequent in SIDS found dead prone ($p = 0.006$ for both) (38)

[a] *Number of cases.*

Genes and SNPs with no significant findings are given in ***Table 3***.

and SNP genotypes, an association between the gene combination IL-1α VNTR A1A1/ +4845TT and SIDS was disclosed, 16% of the SIDS cases had this combination compared to 1.8% of the controls ($p < 0.01$) (**Table 2**). In the SIDS group, it was also found that the genotypes IL-1β −511CC/CT were significantly more frequent in the SIDS victims who found dead in a prone sleeping position compared with SIDS victims who found dead in other sleeping positions ($p = 0.004$) (39).

The 89bp VNTR in the IL-1Ra gene have also been investigated in Australian SIDS cases, where it was found that carriage of the 2/2 genotype increased the risk for SIDS compared with the predominant 1/1 genotype (**Table 2**) (41). Homozygous carriers of allele 2 show a more severe and also prolonged pro-inflammatory immune response compared to other IL-1Ra genotypes (49), which may contribute to the vulnerability to infection seen in SIDS. A smaller study investigating the IL-1ra VNTR in 13 SIDS cases and 103 controls found an association between A2A2 and SIDS (**Table 1**) (42).

TUMOR NECROSIS FACTOR ALPHA

Tumor necrosis factor alpha (TNF-α) is a transmembrane protein produced as a result of the presence of bacterial toxins. TNF-α is an important regulator of immune cells, in addition to stimulate inflammation and controlling viral replication.

Table 3 | Genes and SNPs with no significant findings with regard to SIDS.

Gene	Variant	SIDS[a]	Controls[a]	Findings, reference
IL-1β	−511C/T	93	122	(40)
	−511C/T	204	131	(39)
TGF-β	+869C/T	23	330	Underpowered study (29)
	+869T/C	25	136	Underpowered study (35)
IFN-γ	+874A/T	25	136	Underpowered study (35)
	+874A/T	148	131	(38)
	+874A/T	69	221	No association to SIDS, but strong tendency ($p = 0.06$) (48)
IL-4	−590C/T	25	136	Underpowered study (35)

[a] *Number of cases.*

Two smaller studies have investigated the TNF-α polymorphisms −238A/G and −308A/G, but did not find any association to SIDS (**Table 2**) (29, 32). Five SNPs in the promoter region of the TNF-α gene have been investigated in a Norwegian SIDS population (43). The study found an association between the genotype −238GG and SIDS (**Table 2**). In addition, SNP profiles −1031CT/−238GG/−857CC/−308GG and −1031TT/−238GG/−857CC/−308AA found more often in SIDS; this may therefore be unfavorable SNP combinations (43). An Australian study investigated the −308G/A polymorphism in SIDS cases from different countries, and found a significantly higher proportion of the AA genotype among Australian SIDS cases compared to controls (**Table 2**) (44).

OTHER CYTOKINES

In addition to the genes mentioned above have also SNPs in the genes encoding IL-4 IL-8, IL-12, IL-13, IL-16, IL-18, TGF-β1, and INF-γ been investigated in SIDS (29, 35, 38). A Norwegian study found that the genotypes IL-8 −251AA/AT and IL-8 −781CT/TT were significantly more frequent in SIDS cases found dead in a prone sleeping position compared with SIDS cases found dead in other sleeping positions (**Table 2**) (38). Further, the IL-8 genotypes −251AA/AT and −781CT/TT were more often observed in SIDS cases with positive HLA-DR and one or more risk factors compared with SIDS cases with negative HLA-DR, no infection, and supine sleeping (45). An Australian study investigating SIDS cases from different countries found a marginal association with the IFN-γ +874AA genotype and SIDS (**Table 3**) (48).

OTHER IMMUNE GENES

An increased vulnerability to infection may also be due to genetic variation in the genes encoding G-proteins. The most investigated polymorphism in the Gβ3 gene is C825T, and it is shown that T-allele results in increased G-protein-mediated signal transduction compared to the C-allele (50). Most interleukin-receptors are G-protein coupled, and an association between the Gβ3 825T allele and increased cell function has been reported (51). A study looking at the C825T polymorphism in SIDS victims, cases of infectious death, and live infant controls, revealed no difference in genotype frequency between SIDS cases and controls (52), but an association between the CC genotype and infectious death was found ($p = 0.016$). This observation may indicate that the presence of the 825T allele exerts a protective effect toward serious infection, perhaps through enhanced G-protein signaling.

Surfactant protein A and surfactant protein D (SP-D) are humoral molecules involved in the innate host defense against various bacterial and viral pathogens. Ten common SNPs that might influence expression of the genes encoding these two surfactants have been investigated in SIDS cases and controls ($p = 0.08$) (53). No difference in genotype distribution was found, even though there was a tendency for the most common SP-A haplotype, 6A2/1A0, to be overrepresented in cases with low immunohistochemical SP-A expression (53). None of the other SP-A haplotypes was associated with high or low SP-A expression, and the same was true for the two investigated SP-D SNPs (53).

Polymorphisms that influence the expression of toxin receptors could contribute to SIDS, at least in the cases where there is evidence of toxin involvement. One gene that influences the expression of receptors for staphylococcal enterotoxin B and C in humans are the TCRBV3S1 gene, and a C–T SNP in a recombination signal sequence (RSS) gene region is shown to influence the expression of the gene (54). This SNP have been investigated in 48 Australian SIDS cases and 96 controls, but no differences were found between SIDS cases and controls (55).

Another protein that might be of importance with regard to endotoxins in SIDS is CD14. The TT genotype of the CD14 −260C/T polymorphism causes a significantly higher density of CD14 receptor expression in monocytes, which makes the individual more sensitive to endotoxin than those with the CC genotype (56). This polymorphism has been investigated in an Australian cohort of 116 SIDS cases and 228 controls (57). No differences were found in genotype frequencies between SIDS cases and controls, and the authors conclude that the CD14 −260C/T polymorphism is unlikely to be involved in SIDS (57).

DISCUSSION

The many genetic studies within immune genes in SIDS strongly suggest that these infants do have a combination of genotypes making them vulnerable to infections. The predispositions reported so far are mostly common SNPs with association to SIDS, but genetic variants with strong dominance in SIDS still remains to be uncovered. However, the challenges within genetic studies of SIDS are many. First and foremost is the fact that the SIDS diagnosis is an exclusion diagnosis and important challenge, and even though a great effort has been done in order to standardize is still different diagnostic criteria used in different countries. This might result in that infant with other causes of death, such as accidental asphyxia, or interstitial pneumonia might be included in the SIDS group, and thus camouflage a true genetic association.

Another challenge to genetic studies is the genetic variation between different ethnic groups, which makes it difficult to compare results between different studies without including a potential error. This must be taken into account when investigating SIDS. In addition, the investigated SIDS populations are often small, which make the genetic studies more difficult to interpret. The number of SIDS cases a year in, for instance, Norway is at the moment

about 20, making it difficult and time consuming to be able to collect a large number of cases.

The fact that the number of cases and controls in each study often is small in relation to the number of statistical tests that is undertaken increases the risk of false negatives. Several studies include <25 SIDS cases, which makes it difficult to draw any firm conclusions. Even so, to our best of knowledge are all studies investigating immune genes in SIDS included in **Tables 1–3**, in order to give a comprehensive survey of the research done regarding this topic so far. A large scale GWAS study might be an important next step. The drawback of this method is, however, that important information may be lost due to the corrections that have to be made when investigating such a vast number of polymorphisms, in addition to the problem with limited number of cases. Even so, one might find polymorphisms that could be expected to have a small effect on the SIDS risk. This may be important as these gene variants may point directly to the underlying cause, such as for instance a sub-optimal immune reaction.

Despite the limitations, association studies are still able to report several genetic variations within many immune genes that might be associated with SIDS (**Tables 1** and **2**). This strongly suggest that these genes are of importance and hopefully in the future can provide even better and more accurate understanding of the role of the immune system in SIDS. However, immunology is a complicated biological field, and perhaps the most interesting frontiers in the genetics of immunity arise in the interaction between immune activity and other physiological or developmental processes. A better understanding of how genetic variation might influence the functional effect of the encoded proteins and how this can lead to a fatal outcome is important, and hopefully, with the new possibilities present within genetic research, this can be elucidated. Further would an in-depth analysis of the correlation between genotypes, interleukin response, and risk factors, such as smoking exposure, in different SIDS populations be interesting and might shed light on the involvement of the immune response in these deaths.

CONCLUSION

The studies so far suggest that some SIDS infants may have a genetic vulnerability in the regulation of the immune system. An unfortunate combination of polymorphisms in genes involved in the immune system, in particular in the cytokine genes, may lead to an imbalance in the immune response and render the infant unable to cope with an infection. However, the immune system and the cytokine network are complex, and more studies are needed in order to better understand the interplay between different genetic variations and how this may contribute to an unfavorable immunological response. The associations observed so far between different polymorphisms and SIDS and environmental factors for SIDS most likely represent only a small part of genetic patterns that may result in an unfortunate immunological reaction.

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Virus infections and sudden death in infancy: the role of interferon-γ

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Respiratory infections have been implicated in sudden infant death syndrome (SIDS). As interferon-γ (IFN-γ) is a major response to virus infection, we examined (1) the frequency of single nucleotide polymorphism (SNP), *IFNG* T + 874A, in SIDS infants, their parents, and ethnic groups with different incidences of SIDS; (2) model systems with a monocytic cell line (THP-1) and human peripheral blood monocytes (PBMC) for effects of levels of IFN-γ on inflammatory responses to bacterial antigens identified in SIDS; (3) interactions between genetic and environmental factors on IFN-γ responses. *IFNG* T + 874A genotypes were determined for SIDS infants from three countries; families who had a SIDS death; populations with high (Indigenous Australian), medium (Caucasian), and low (Bangladeshi) SIDS incidences. The effect of IFN-γ on cytokine responses to endotoxin was examined in model systems with THP-1 cells and human PBMC. The IFN-γ responses to endotoxin and toxic shock syndrome toxin (TSST-1) were assessed in relation to genotype, gender, and reported smoking. There was a marginal association with *IFNG* T + 874A genotype and SIDS ($p = 0.06$). Indigenous Australians had significantly higher proportions of the *IFNG* T + 874A SNP (TT) associated with high responses of IFN-γ. THP-1 cells showed a dose dependent effect of IFN-γ on cytokine responses to endotoxin. For PBMC, IFN-γ enhanced interleukin (IL)-1β, IL-6, and tumor necrosis factor-α responses but reduced IL-8 and IL-10 responses. Active smoking had a suppressive effect on baseline levels of IFN-γ. There was no effect of gender or genotype on IFN-γ responses to bacterial antigens tested; however, significant differences were observed between genotypes in relation to smoking. The results indicate virus infections contribute to dysregulation of cytokine responses to bacterial antigens and studies on physiological effects of genetic factors must include controls for recent or concurrent infection and exposure to cigarette smoke.

Keywords: sudden infant death syndrome, IFN-γ, ethnicity, cigarette smoke, cytokines

INTRODUCTION

The peak in sudden infant death syndrome (SIDS) occurs during the developmental period in which infants have low levels of specific antibody protection, either maternal or actively acquired immunity. They are dependent on their innate responses to deal with new infectious agents they encounter in their environment. Infection and inflammatory responses have been implicated in many of these deaths and we have examined the hypothesis that some SIDS deaths occur as a result of dysregulation of inflammatory responses which can affect physiological systems suggested to be involved in triggering these deaths (1).

There is a growing body of evidence that infection (1–3) and the inflammatory response to these infections (1, 4, 5) play a role in both SIDS and sudden unexpected death in infancy (SUDI). Inflammatory changes, particularly in the respiratory tract, are common findings in SIDS. It has been suggested that these findings reflect recent infections, symptoms of which have been noted in the 2 weeks prior to death for over 40% of SIDS infants (6, 7). Mild respiratory infections have been noted prior to death, but no single virus has been implicated (8). In infants, virus infections have been demonstrated to enhance the number and variety of bacterial species in the nasopharynx (9), particularly among infants sleeping in the prone position. While specific virus infections were not identified among SIDS infants, it was noted that there was a significantly higher proportion of coliform organisms in the respiratory tract of many (10). Both *Staphylococcus aureus* and *Escherichia coli* were identified in a significantly higher proportion of SUDI infants than infants who died of known causes (2, 3).

In animal models, induction of pro-inflammatory cytokines such as interferon-γ (IFN-γ) contribute to the severity of a host's responses to either infectious agents or their products, and this can be greatly enhanced by a co-existing virus infection (11–14). Toxigenic bacteria or their toxins have been implicated in the etiology of SIDS (15–20). Priming with an asymptomatic virus

infection can significantly reduce the concentration of bacterial toxins needed to induce death.

There have been reports that levels of IFN-γ responses differ between the single nucleotide polymorphisms (SNPs) genotypes (21, 22). We have demonstrated in previous studies that these can vary among different ethnic groups (23) and that there are interactions between genotypes and exposure to environmental agents such as cigarette smoke (24). As virus infections and exposure to cigarette smoke are both significant risk factors for SIDS, our study tested the following hypotheses: (1) the single nucleotide polymorphism *IFNG* T + 874A genotype associated with higher IFN-γ responses might be over-represented among SIDS infants or ethnic groups in which there is a higher incidence of SIDS; (2) as a surrogate for virus infection, exposure to IFN-γ would significantly alter cytokine responses from human leukocytes to bacterial antigens identified in SIDS infants; (3) cigarette smoking might affect IFN-γ responses to bacterial toxins *in vitro*, as noted previously for other cytokines (24, 25).

SUBJECTS AND METHODS

Approval for the study was obtained from the Lothian Health Ethics Committee (UK), Hunter Area Research Ethics Committee (Australia), and the University of Newcastle Human Research Ethics Committee (Australia).

ASSESSMENT OF *IFNG* T + 874A SNP

Buccal epithelial cells were collected from Caucasian parents of SIDS infants from Britain ($n = 34$) and Australia ($n = 60$), and their matched controls with no family history of SIDS (Britain $n = 59$, Australia $n = 55$).

Paraffin-fixed samples of tissue from SIDS infants were obtained from Australia ($n = 17$), Hungary ($n = 21$), and Germany ($n = 47$). Stored frozen whole blood samples from Indigenous Australians ($n = 123$) and buccal epithelial cells from Bangladeshis ($n = 32$) were used as DNA sources for comparisons between ethnic groups. The methods for extraction of DNA from the samples have been described previously (24–26).

To genotype *IFNG* T + 874A (rs2430561) a custom made allelic discrimination polymerase chain reaction (PCR) assay was manufactured (PE Applied Biosystems). Primers: 5′ GCT GTC ATA ATA ATA TTC AGA CAT TCA CAA TTG AT 3′; 5′ TGC GAG TGT GTG TGT GTG T 3′ and probes: 5′ CAC AAA ATC AAA TCT CAC ACA C 3′; 5′ ACA AAA TCA AAT CAC ACA CAC 3′ were provided in a 40× assay mix.

Each PCR reaction contained 10 ng of sample DNA, 1× Assay mix, and 1× TaqMan Universal PCR Master Mix (PE Applied Biosystems) made up to a final volume of 5 μl with sterilized MilliQ water. PCR was performed using the ABI PRISM 7900HT sequence detection system (PE Applied Biosystems) at the following thermal cycling conditions: 50°C for 2 min; 95°C for 10 min; 92°C for 15 s; and 60°C for 1 min, for 40 cycles.

Data were analyzed using the statistical software package Statistics/Data Analysis™(STATA) Version 8.0 (Stata Corporation, College Station, TX, USA). The Chi-square (χ^2) test or Fisher's exact test, if appropriate, was used to assess the distribution of *IFNG* T + 874A in SIDS infants, parents of SIDS infants, and between ethnic groups. Deviation of *IFNG* T + 874A genotype distribution from Hardy–Weinberg equilibrium (HWE) was assessed using the χ^2 test.

ASSESSMENT OF THE EFFECTS OF IFN-γ ON RESPONSES TO BACTERIAL ANTIGENS

The THP-1 human monocytic cell line used in previous studies of inflammatory responses to bacterial endotoxin (27) was used in preliminary studies to determine concentrations of components to be used in the model system (28).

Collection, isolation, and storage of human peripheral blood monocytic cells (PBMC) have been described previously (29). Buffy coats from 14 male ($n = 14$) and 14 female ($n = 14$) donors, aged 20–55 years, were purchased from the Australian Red Cross Blood Service (ARCBS) (Sydney, NSW, Australia). Ethical permission was obtained from University of Newcastle Human Research Ethics Committee (H-229-0606) and ARCBS Ethics Committee (07-11NSW-07) for the purchase and use of human buffy coats for the purposes of the study. PBMC were collected from each donor for *in vitro* cytokine stimulation assays and plasma was collected for the assessment of cotinine for evidence of exposure to cigarette smoke, a confounding variable for altered cytokine responses. Donors with detectable levels of cotinine were excluded from the analysis. Only ARCBS donor samples that were cleared for infectious agents were received. Buffy coats were processed within 24 h of collection.

Blood samples (10–20 ml) were collected from British parents of SIDS infants ($n = 34$) and control individuals ($n = 59$) who had no family history of SIDS. Leukocytes were isolated and stored as described previously (24–26).

ANALYSIS OF EXPOSURE TO CIGARETTE SMOKE

As described previously (29), plasma from the samples from ARCBS donors were assessed for exposure to cigarette smoke by a semi-quantitative commercial competitive enzyme immunoassay (EIA) kit according to manufacturer's instructions (OraSure Technologies Inc., Bethlehem, PA, USA). To prevent false negative classification of exposure to cigarette smoke, the qualitative cut-off of the assay was lowered from the recommended 25 to 10 ng ml^{-1}. Donors with detectable levels of cotinine were excluded from stimulation assays.

STIMULATION ASSAYS

Conditions for experiments with the THP-1 cells have been described previously (28).

Peripheral blood monocytic cells from the ARCBS donors ($n = 28$) were assessed for *in vitro* cytokine responses to a common bacterial antigen, *E. coli* lipopolysaccharide (LPS) (50 ng ml^{-1}). IFN-γ (10 ng ml^{-1}) was used as a surrogate for virus infection. A water-soluble cigarette smoke extract (CSE) was used as a surrogate for exposure to cigarette smoke. The method has been described previously (29).

The leukocytes from British parents of SIDS infants were assessed for inflammatory responses to TSST-1 and LPS as described previously (24–26). All samples were coded and tested without knowing the smoking status of the donors. Leukocytes (1×10^6 cells ml^{-1}) were stimulated *in vitro* with 0.01, 1 μg ml^{-1} *E. coli* endotoxin or 0.5 μg ml^{-1} toxic shock syndrome

toxin (TSST-1) (Sigma, Poole, Dorset, UK) for 24 h. Cell culture conditions have been described previously (24–26).

ANALYSIS OF CYTOKINE RESPONSES

Supernatants from the THP-1 and PBMC assays were measured for IL-1β, IL-6, tumor necrosis factor-α (TNF-α), IFN-γ, IL-8, and IL-10 using Bio-Rad 6-plex assays and the Luminex 200 analyzer. Cytokine concentrations (pg ml^{-1}) were calculated from the standard curve using the Luminex 2.3 Software. Data were analyzed using STATA™ Version 10.0. Mean donor cytokine measurements were used for statistical analysis. The Wilcoxon matched-pairs signed ranks test was used to assess differences in cytokine responses between treatment groups. The significance level was set at $p < 0.05$.

Supernatants from assays with leukocytes from the British SIDS and control families were measured for IFN-γ levels using enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Results were expressed as ng ml^{-1} derived from the standard curves obtained using a recombinant human IFN-γ standard (R&D Systems). The result for each group assessed was reported as the median value. Student's *t*-test was used on log-transformed data to assess differences in IFN-γ responses of smokers and non-smokers and of males and females in relation to genotype. The significance level for all tests was set at $p < 0.05$.

RESULTS

ANALYSIS OF GENOTYPES OF *IFNG* T + 874A

The distributions of *IFNG* T + 874A genotypes among the different groups assessed are summarized in **Table 1**. Distributions were in HWE for each of the groups assessed, except for the Australian SIDS infants.

DISTRIBUTION OF *IFNG* T + 874A AMONG SIDS INFANTS

There were no significant differences in the *IFNG* T + 874A distribution in SIDS infants from different countries (**Table 1**). The predominant genotype among Australian SIDS infants was AA (9/16, 56%); approximately half of Hungarian (4/8, 50%) and German (21/45, 47%) infants possessed the TA genotype.

The distribution of *IFNG* T + 874A in the Australian control population approached a significant difference from that observed for Australian SIDS infants ($p = 0.06$). The genotype distribution of the Australian SIDS infant group was inversely proportional to the distribution of the control population. There was a predominance of the TT (25%) and AA (56%) genotypes, and an under representation of the TA (19%) genotype. The Australian control population was similar to all other populations, with approximately half the population (51%) with the TA genotype. The under representation of the TA genotype in the Australian SIDS infant group caused the genotype distribution to be significantly different from that of the HWE ($p = 0.02$). No significant differences were detected between the distributions of *IFNG* T + 874A genotype for the combined SIDS infant group compared to the Caucasian controls ($p = 0.52$).

ASSESSMENT OF *IFNG* T + 874A AMONG PARENTS OF SIDS INFANTS

Differences in the distribution of *IFNG* T + 874A genotype among parents of SIDS infants were not statistically significant compared to their respective control populations (**Table 1**). Parents of SIDS infants recruited from Britain showed an increased proportion of individuals with the TA genotype compared to the matched British controls but this was not significant ($p = 0.53$). Parents of SIDS infants recruited from Australia had a similar distribution to their control population, with the majority of individuals possessing the TA genotype ($p = 0.77$).

Table 1 | *IFNG* T + 874A allele frequency distributions across populations.

Ethnicity	Group		Allele frequency (%)			Sample size (*n*)	*p* Value (*p*)
			TT	TA	AA		
British	SIDS	Parents	12	56	32	34	0.53
British	Control	Parents	14	44	42	59	
Australian	SIDS	Parents	13	55	32	60	0.77
Australian	Control	Parents	18	51	31	55	
Australian	SIDS	Infants	25	19	56	16	0.06[a]
Hungarian	SIDS	Infants	13	50	38	8	0.89[b] 0.39[c]
German	SIDS	Infants	22	47	31	45	0.12[d]
Combined	SIDS	Infants	22	41	38	69	0.52[e]
Bangladeshi	Control		13	50	38	32	0.93[f]
Indigenous Australian	Control		39	49	12	75	<0.01[g] <0.01[h]
Caucasian	Control		16	47	37	114	

[a] *Australian SIDS infants vs. Australian control parents.*
[b] *Hungarian SIDS infants vs. German SIDS infants.*
[c] *Hungarian SIDS infants vs. Australian SIDS infants.*
[d] *German SIDS infants vs. Australian SIDS infants.*
[e] *Combined SIDS infants vs. Caucasian control.*
[f] *Bangladeshi control vs. Caucasian control.*
[g] *Indigenous Australian vs. Caucasian control.*
[h] *Indigenous Australian vs. Bangladeshi control.*

DISTRIBUTION OF *IFNG* T + 874A IN DIFFERENT ETHNIC GROUPS

As there were no differences between the distribution of *IFNG* T + 874A genotype for British and Australian control populations, these data were combined for further comparison with samples from ethnic groups with low incidences of SIDS (Bangladeshi) or high incidences (Indigenous Australian) compared with Caucasian populations. The majority of each population possessed the TA genotype. The distribution of *IFNG* T + 874A genotype was similar for the Caucasian and Bangladeshi populations; however, both were significantly different from the Indigenous Australian population ($p < 0.01$) (**Table 1**). The Aboriginal Australian population had a significant increase in the proportion of the individuals with the TT genotype compared to the Caucasian and Bangladeshi populations.

EFFECTS OF IFN-γ AND CSE ON CYTOKINE RESPONSES ELICITED BY LPS FROM THP-1 CELLS

In the preliminary experiments with THP-1 cells, two concentrations of IFN-γ were tested (1 and 10 ng ml^{-1}) to assess the effect of dose on pro-inflammatory responses to LPS. TSST was not

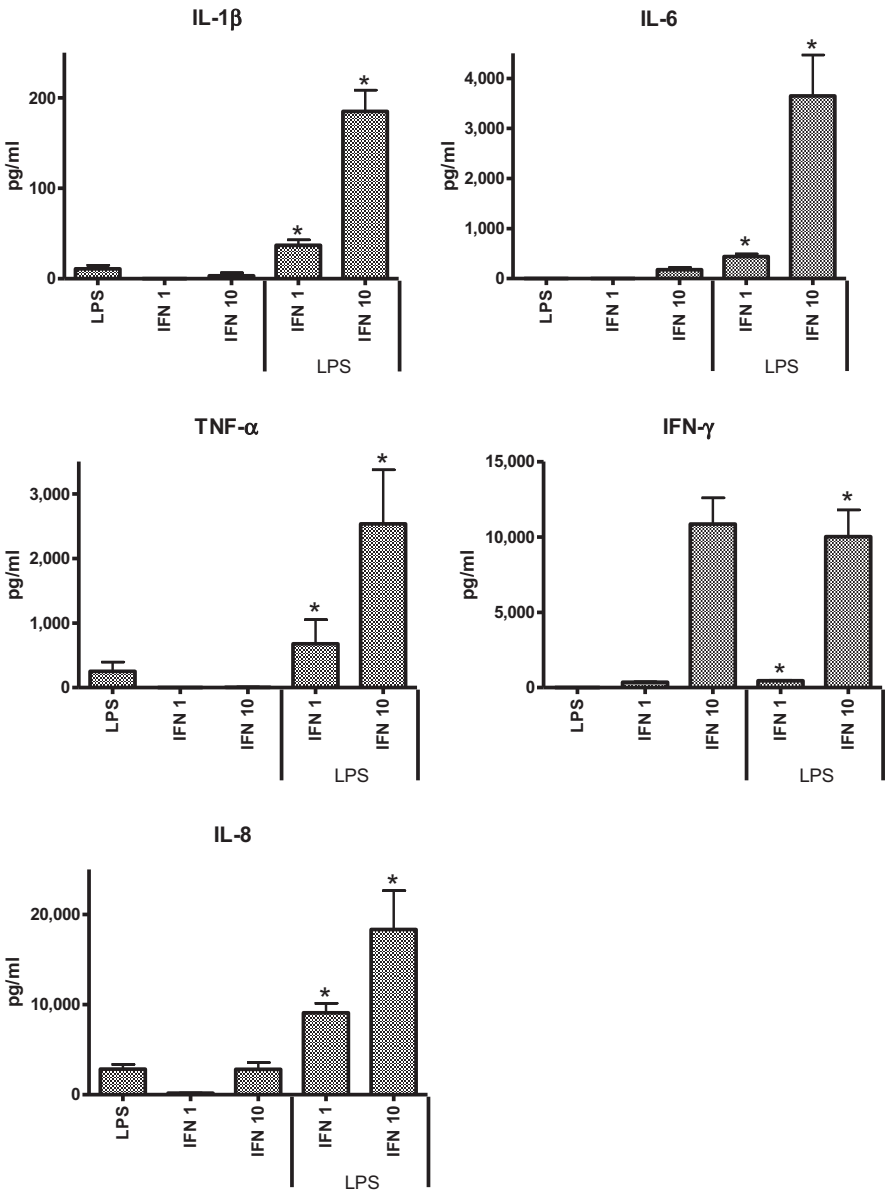

FIGURE 1 | Effects of IFN-γ, 1 ng ml^{-1} (IFN 1) or 10 ng ml^{-1} (IFN 10) on cytokine responses of THP-1 cells to LPS (50 ng ml^{-1}).

assessed in these preliminary studies as previous work indicated that THP-1 cells did not respond to the toxin. IL-6 was not elicited from THP-1 cells in the absence of IFN-γ. The higher concentration of IFN-γ induced significantly higher levels of IL-1β, IL-6, and TNF-α (**Figure 1**). The anti-inflammatory IL-10 was not detected under any of the conditions tested.

Because our previous studies indicated cigarette smoke exposure could significantly affect cytokine responses in the model systems tested (24), we assessed the effects of CSE alone or CSE and IFN-γ on responses to LPS. Pre-treatment of THP-1 cells with CSE resulted in decreased responses to LPS. Although the higher dose of CSE (0.001 cigarette ml^{-1}) appeared to reduce IL-1β, TNF-α, and IFN-γ responses, the effect was not significant. Both low and high concentrations of CSE-enhanced IL-8 responses, but these were not significant. CSE exposure did not have a significant effect on responses induced with LPS from IFN-γ primed THP-1 cells.

EFFECTS OF IFN-γ AND CSE ON CYTOKINE RESPONSES FROM PBMC

The PBMC from 28 donors were used to assess the effect of priming with 10 ng ml^{-1} IFN-γ on cytokine responses to LPS. A similar pattern of enhancement of pro-inflammatory responses elicited by the IFN-γ-primed cells was observed with PBMC from blood donors. CSE exposure reduced the effects of IFN-γ priming on LPS stimulation of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. In contrast to results with THP-1 cells, IFN-γ priming significantly reduced IL-8 responses and the anti-inflammatory

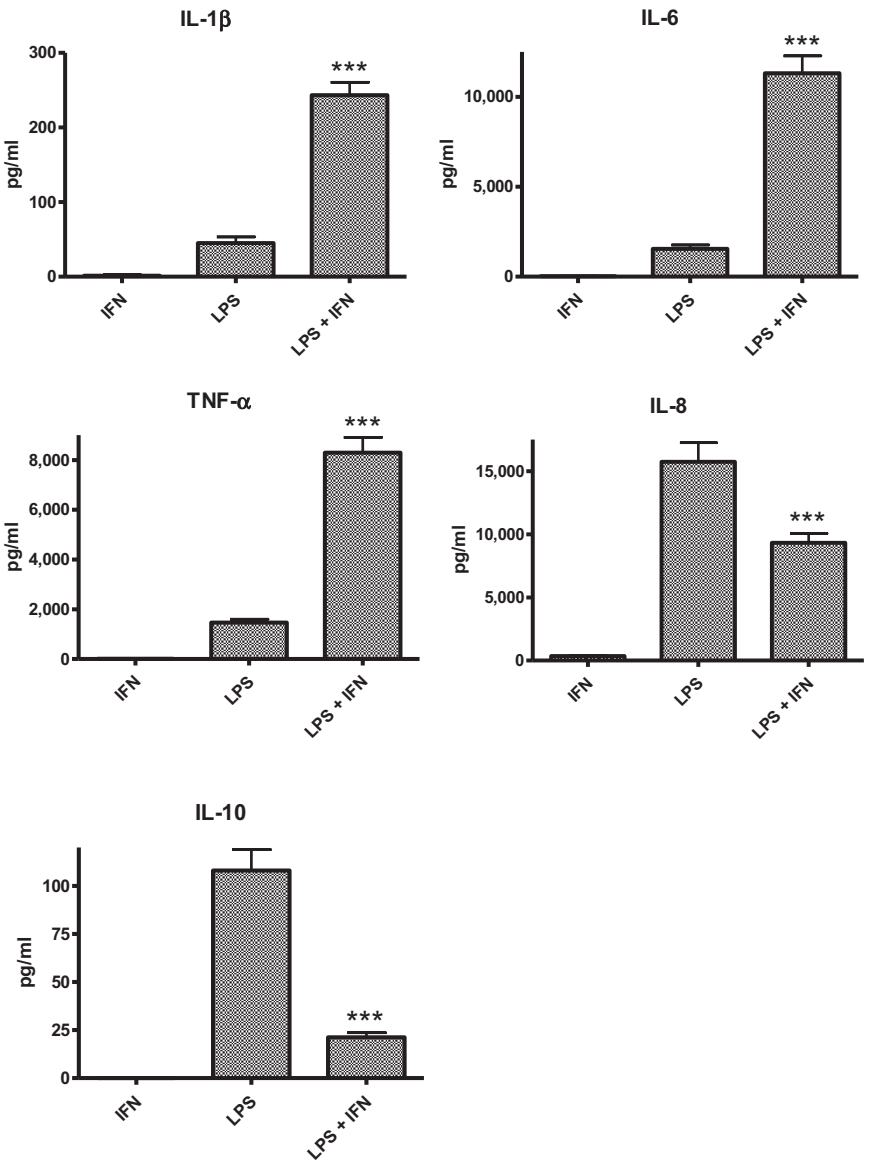

FIGURE 2 | The effect of IFN-γ (10 ng ml^{-1}) pre-treatment on LPS (50 ng ml^{-1}) stimulation of IL-1β, IL-6, TNF-α, IL-8 and IL-10 from PBMC ($n = 28$) ***$p < 0.0001$.

IL-10 responses of PBMC to LPS (**Figure 2**). LPS stimulated cells pre-treated with both IFN-γ and CSE had the lowest responses for both IL-8 and IL-10 (**Figure 3**).

EFFECT OF SMOKING, GENOTYPE, AND GENDER ON IFN-γ RESPONSES TO LPS OR TSST-1

The assays with PBMC from SIDS parents and unrelated individuals were used to assess the effects of smoking and genotype on IFN-γ responses to LPS or TSST-1. The results are summarized in **Table 2** and described below.

Smoking

Smoking had a small but significant suppressive effect on baseline IFN-γ levels (1 ng ml^{-1}) compared to non-smokers (4.15 ng ml^{-1}) ($p < 0.01$). This effect disappeared when cells were stimulated with 0.01 or 1 μg ml^{-1} LPS or 1 μg ml^{-1} TSST.

Genotype

When analyzed irrespective of smoking status, there were no significant differences between IFN-γ responses among *IFNG* T + 874A genotypes for either of the stimulants.

Gender

Overall, there were no differences in IFN-γ responses of cells from males compared with those from females. When smoking status was assessed in relation to gender, there was a significant difference in the IFN-γ response to 1 μg ml^{-1} TSST-1 for non-smokers: males (567 ng ml^{-1}); females (1,167 ng ml^{-1}) ($p = 0.01$). This

trend was also observed for 1 μg ml^{-1} LPS; however, differences were not significant ($p > 0.05$).

INTERACTIONS BETWEEN GENOTYPE AND SMOKING IN RELATION TO IFN-γ RESPONSES TO LPS

The IFN-γ responses of cells from donors with the TT genotype to 0.01 μg ml^{-1} LPS was significantly decreased in smokers (1.85 ng ml^{-1}) compared to non-smokers (145.8 ng ml^{-1}) ($p = 0.02$). For non-smokers, the reverse pattern was observed; IFN-γ responses of TT donors were higher than those of the other genotypes but the differences were not significant. The IFN-γ responses to 0.01 μg ml^{-1} LPS from cells of smokers with the TT genotype (1.85 ng ml^{-1}) was significantly lower compared to those from cells of smokers with the TA (69.9 ng ml^{-1}) ($p < 0.01$) or AA genotypes (75.1 ng ml^{-1}) ($p = 0.01$).

INTERACTIONS BETWEEN GENOTYPE AND SMOKING IN RELATION TO IFN-γ RESPONSES TO TSST

Smokers had lower baseline IFN-γ levels than non-smokers. For individuals with the AA genotype, smokers had significantly lower IFN-γ responses (1.85 ng ml^{-1}) compared to non-smokers (6.6 ng ml^{-1}) ($p = 0.03$).

Differences between genotypes were observed for smokers when cells were stimulated with 1 μg ml^{-1} TSST. Cells from smokers with the TA genotype (2,039 ng ml^{-1}) had significantly higher IFN-γ responses than those with the TT (1,039 ng ml^{-1}) ($p = 0.03$) or AA genotypes (1,292 ng ml^{-1}) ($p = 0.05$) (**Table 2**).

When genotypes were assessed in relation to smoking status, there was a significant difference for the TA genotype. Cells from smokers had higher IFN-γ responses to 1 μg ml^{-1} TSST (2,039.7 ng ml^{-1}) compared to non-smokers (1,136.1 ng ml^{-1}) ($p = 0.01$).

DISCUSSION

We found no significant associations with the *IFNG* T + 874A SNP genotypes and SIDS. As found for the previous report on a British population (30), there was a marginal difference between the SIDS and local control groups ($p = 0.06$) in the distribution of the *IFNG* T + 874A genotype; however, the distribution of the genotypes between European and Australian SIDS infants was different. Among the European infants, the TA genotype was predominant; but among the Australian infants the AA genotype was predominant (56%).

It is not unusual for cytokine gene polymorphisms to differ between countries, particularly among disease cohorts. Differences between countries reinforce the need for local controls and caution exercised for interpretation of differences between disease and control groups from different ethnic backgrounds and countries.

Due to the under representation of the TA genotype, the genotype distribution of the Australian SIDS infant group was out of HWE. This was observed for two other SNPs previously assessed in this population; *IL1RN* T + 2018C and *IL10* G-1082A (24, 26). The decrease in the heterozygous genotype of *IFNG* T + 874A, *IL1RN* T + 2018C, and *IL10* G-1082A in the Australian SIDS infant population indicates that there is bias in the selection of the population. This might highlight the importance of these SNPs in the etiology of SIDS in Australia.

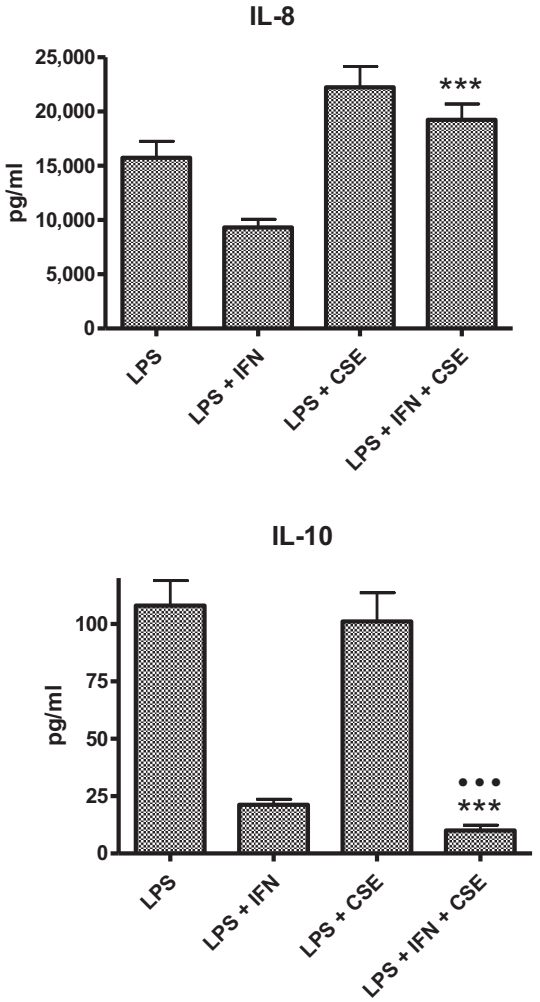

FIGURE 3 | Combined effects of IFN-γ and CSE on IL-8 and IL-10 responses of PBMC to LPS ($n = 28$) *** $p < 0.0001$.

This study has again demonstrated the differences in distributions of cytokine gene polymorphisms among ethnic groups. There is an increased proportion of individuals with *IFNG* T + 874A TT genotype in the Indigenous Australian population compared to Caucasian and Bangladeshi populations. No Indigenous Australian SIDS infants were examined in the study.

In general, the effect of IFN-γ was similar for both THP-1 and PBMC models, enhancing pro-inflammatory responses IL-1β, IL-6, and TNF-α to stimulation with LPS. In contrast to results obtained with THP-1 cells, IFN-γ significantly reduced IL-8 responses of PBMC to LPS. Addition of CSE to the THP-1 cells primed with IFN-γ did not significantly alter the responses compared with priming cells with IFN-γ only. Addition of CSE to the PBMC primed with IFN-γ reduced the enhanced IL-1β, IL-6, and TNF-α responses to LPS. IFN-γ significantly reduced the CSE-enhanced IL-8 responses to LPS. Potentially, the most significant physiological effect was the reduction in IL-10 responses in the presence of both IFN-γ and CSE (**Figure 3**). If the effects

Table 2 | Median IFN-γ levels of 1 × 10^6 cells/ml leukocytes stimulated *in vitro* with TSST and endotoxins (LPS), assessed by *IFNG* T + 874A genotype and smoking status.

Smoking status	*IFNG* + 874A	Gender	Median IFN-γ response (ng/ml) to toxin (μg/ml)				Sample size (*n*)
			Baseline	TSST-1 1	LPS 1	LPS 0.01	
Smoker	All	All	1	1,542	467	55	45
Non-smoker			4	1,074	559	88	74
All	TT	All	1	895	821	51	15
	TA		1	1,357	613	83	48
	AA		5	1,452	477	90	41
All	All	Male	1	893	874	88	26
		Female	1	1,135	620	57	52
Smoker	TT	All	1	1,039	440	2	4
	TA		1	2,040	550	70	11
	AA		2	1,293	468	75	17
Non-smoker	TT		3	895	928	146	31
	TA		3	1,136	623	86	18
	AA		7	1,477	525	104	23
Smoker	All	Male	1	1,950	1,120	94	10
Non-smoker			3	567	490	88	16
Smoker		Female	1	927	490	48	19
Non-smoker			5	1,167	638	69	33
Smoker	TT	Male	1	2,857	821	3	1
	TA		1	2,121	2,260	432	4
	AA		1	1,822	359	16	3
Non-smoker	TT		1	215	812	158	2
	TA		1	647	406	83	9
	AA		386	550	570	421	2
Smoker	TT	Female	1	1,039	607	12	2
	TA		2	808	340	51	6
	AA		5	1,293	664	75	10
Non-smoker	TT		6	1,818	1,411	214	6
	TA		1	1,151	1,243	51	10
	AA		7	831	477	69	15

of these two risk factors for SIDS demonstrated *in vitro* reflect the responses to infection *in vivo*, the combined effects of cigarette smoke and virus infection on reduction of IL-10 could result in significant dysregulation of pro-inflammatory responses. We have previously demonstrated a dose-dependent reduction in pro-inflammatory responses in our model system with increasing levels of IL-10 (24).

Genotype alone was not responsible for differences in IFN-γ responses. When variables were assessed independently, few differences were observed. Smoking had a small, but suppressive effect on baseline IFN-γ levels. There was no effect of gender or *IFNG* T + 874A genotype on IFN-γ responses.

When the effects of smoking status and gender on IFN-γ responses were assessed, the only difference between genders was among non-smokers; males had reduced IFN-γ response to TSST-1. Gender differences in cytokine responses have been previously described (29, 31, 32) and are thought to be associated with the respective levels of sex hormones. Our findings correspond with the majority of the literature; there were higher pro-inflammatory responses in females compared to males.

When the effects of smoking status and *IFNG* T + 874A genotype on IFN-γ levels were assessed, significant differences were observed between genotypes among smokers. In general, the effect of smoking was suppressive. The most dramatic effect was the 10-fold decrease in the response to the lower dose of endotoxin in smokers with the TT genotype compared to non-smokers. The only increase in IFN-γ responses in those who smoked was in individuals with the TA genotype when cells were stimulated with TSST-1. This was the most common genotype observed among SIDS infants from Hungary and Germany (**Table 1**).

The interaction of cigarette smoking with *IFNG* T + 874A genotypes on IFN-γ responses appears to be toxin specific. For example, for TSST, the TA genotype was greatly affected by cigarette smoke, while for endotoxin ($0.01\,\mu\,ml^{-1}$) the TT genotype

was most significantly affected by smoking. It is common to find conflicting data in the literature when assessing the function of a SNP, particularly when stimulation conditions differ. In our study, differences in responses were due to toxin type, as stimulation conditions were the same. We could not, however, control for asymptomatic infection or passive exposure to cigarette smoke as cotinine levels were not assessed in the studies with samples from SIDS parents and the unrelated comparison group.

There is little information in the literature on the effects of the *IFNG* SNP on cytokine responses. Two groups have found the TT genotype to be associated with increased IFN-γ production (22, 33, 34); however, smoking or environmental tobacco smoke (ETS) exposure had not been considered as a confounder. These results again highlight the need to control for smoking status and ETS exposure when assessing the effects of cytokine gene SNPs *in vitro*.

We have observed similar interactions with cytokine gene SNPs and smoking; IL-10 responses were significantly decreased in smokers with the *IL10* G-1082A AA genotype. IL-6 responses were significantly increased in smokers with the *IL6* G-174C GC (24, 25). Data from this study and previous findings suggest that exposure to cigarette smoke alters cytokine responses more for some genotypes than others.

There were significantly higher IFN-γ responses to TSST-1 from cells of donors with the TA genotype, and this was the predominant genotype among SIDS infants from Germany and Hungary (**Table 1**). Pyrogenic staphylococcal toxins have been detected in over 50% of SIDS infants from five different countries (20). Interactions between a mild virus infection and exposure to cigarette smoke in an infant with the TA genotype might lead to high levels of IFN-γ, which could result in significant down regulation of IL-10 and dysregulation of pro-inflammatory responses to pyrogenic toxins. In a vulnerable infant, this dysregulation of inflammation could trigger the physiological events leading to SIDS.

AUTHOR CONTRIBUTIONS

Each of the authors made substantial contributions to the conception, design, analyses, and interpretations of the work. They assisted in preparing the article, critically assessed the final version and agree to be accountable for the accuracy and integrity of the work.

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Genetic and environmental factors affecting TNF-α responses in relation to sudden infant death syndrome

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Dysregulation of the inflammatory responses has been suggested to contribute to the events leading to sudden infant deaths. Our objectives were (1) to analyze a single nucleotide polymorphism (SNP) associated with high levels of tumor necrosis factor-α (TNF-α) responses, *TNF* G-308A, in sudden infant death syndrome (SIDS) infants, SIDS and control parents, and ethnic groups with different incidences of SIDS; (2) the effects of two risk factors for SIDS, cigarette smoke and virus infection, on TNF-α responses; and (3) to assess effects of genotype, cigarette smoke, and gender on TNF-α responses to bacterial toxins identified in SIDS infants. *TNF* G-308A genotypes were determined by real-time polymerase chain reaction for SIDS infants from Australia, Germany, and Hungary; parents of SIDS infants and their controls; and populations with high (Aboriginal Australian), medium (Caucasian), and low (Bangladeshi) SIDS incidences. Leukocytes from Caucasian donors were stimulated *in vitro* with endotoxin or toxic shock syndrome toxin-1 (TSST-1). TNF-α responses were measured by L929 bioassay (IU/ml) and assessed in relation to genotype, smoking status, and gender. There was a significantly higher proportion of the minor allele AA genotype among Australian SIDS infants (6/24, 24%) compared to 3/62 (4.8%) controls ($p = 0.03$). There were no significant differences in TNF-α responses by *TNF* G-308A genotypes when assessed in relation to smoking status or gender. Given the rarity of the *TNF* G-308A A allele in Caucasian populations, the finding that 24% of the Australian SIDS infants tested had this genotype requires further investigation and cautious interpretation. Although non-smokers with the AA genotype had higher TNFα responses to both TSST-1 and endotoxin, there were too few subjects with this rare allele to obtain statistically valid results. No effects of genotype, smoking, or gender were observed for TNF-α responses to these toxins.

Keywords: sudden infant death syndrome, TNF-α, ethnicity, cigarette smoke

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Introduction

Tumor necrosis factor-α (TNF-α) is a key pro-inflammatory cytokine which could play a role in several of the mechanisms proposed to explain sudden infant death syndrome (SIDS). In relation to the anaphylaxis hypothesis, TNF-α is stored in mast cell granules and is released on antigen stimulation. TNF-α also induces fever and affects respiration (1) and can affect myocardial function

(2, 3). One of its single nucleotide polymorphisms (SNPs), *TNF* G-308A, has been associated with severe responses to infection. Cytokine gene polymorphisms can significantly affect the level of these substances produced in response to infection (4). *In vitro* studies found that the A allele was associated with increased responses (5) and individuals homozygous for the A allele had higher levels of circulating TNF-α (6). The molecular basis for the A allele being a more powerful transcriptional activator is not clear; however, the region −323 to −285 has been found to bind nuclear factors differently compared with the corresponding G allele (7).

Reports on the *TNF* G-308A SNP indicate the AA genotype carried an increased risk of death from cerebral malaria (8), septic shock (9), and death from meningococcal sepsis (10). Two studies examined SNPs for TNF-α among Scandinavian SIDS infants and found no significant association (11, 12). Because of the variability of reports on findings for a variety of SNPs among SIDS infants in different populations, we examined the *TNF* G-308A genotypes among material from our collection of samples from Germany, Hungary, and Australia. Because of the variation in the incidence of SIDS among different ethnic groups, we also included comparison groups from populations with low (South Asian), moderate (Caucasian), or high (Indigenous Australian) incidences of SIDS.

Although gene polymorphisms are important determinants of the cytokine responses, we have reported that three major risk factors associated with SIDS – gender, exposure to cigarette smoke, and virus infections – can significantly influence these responses (13–15). In this study, we used model systems to assess interactions between these risk factors and TNF-α responses elicited by components of bacterial species identified in SIDS infants, lipopolysaccharide (LPS) of Gram-negative species (16, 17), and toxic shock syndrome toxin-1 (TSST-1) of *Staphylococcus aureus* (18).

The objectives of the study were (1) to analyze the distribution of *TNF* G-308A alleles among SIDS infants, SIDS parents, an unrelated adult comparison group, and ethnic groups with different incidences of SIDS; (2) to assess effects of virus infection and cigarette smoke on TNF-α responses; and (3) to assess the effect of gender, *TNF* G-308A, and cigarette smoke on responses to bacterial antigens (LPS and TSST-1) identified in SIDS infants.

Subjects and Methods

Approval for the study was obtained from the Lothian Health Ethics Committee (UK), Hunter Area Research Ethics Committee (Australia) and the University of Newcastle Human Research Ethics Committee (Australia).

Assessment of *TNF* G-308A SNP

Buccal epithelial cells were collected from Caucasian parents of SIDS infants from Britain ($n = 34$) and Australia ($n = 60$) and a comparison group with no family history of sudden infant deaths (Britain $n = 56$, Australia $n = 62$). Paraffin-fixed samples of tissue from SIDS infants were obtained from Australia ($n = 25$), Hungary ($n = 18$), and Germany ($n = 46$). Stored frozen blood samples from Aboriginal Australians ($n = 117$) and buccal epithelial cells from Bangladeshi donors ($n = 32$) were used as sources of DNA for comparisons among ethnic groups. The methods for extraction of DNA from the samples have been described previously (13).

TNF G-308A (rs1800629) genotype was determined by a commercial allelic discrimination polymerase chain reaction (PCR) assay (Assay ID: C___7514879_10) (PE Applied Biosystems). Primers and probes were provided in a 20× assay mix, sequences and concentrations of which are unknown.

Each PCR reaction contained 10 ng of sample DNA, 1× Assay mix, and 1× TaqMan Universal PCR Master Mix (PE Applied Biosystems) made up to a final volume of 5 µl with sterilized MilliQ water. PCR was performed using the ABI PRISM 7900HT sequence detection system (PE Applied Biosystems) at the following thermal cycling conditions: 50°C for 2 min; 95°C for 10 min; 92°C for 15 s; and 60°C for 1 min, for 40 cycles.

Data were analyzed using the statistical software package Statistics/Data Analysis™ (STATA) Version 8.0 (Stata Corporation, College Station, USA). The Chi-square (χ^2) test or Fisher's Exact test, if appropriate, was used to assess the distribution of *TNF* G-308A in SIDS infants, parents of SIDS infants, and between ethnic groups. Genotype distribution from the Hardy–Weinberg equilibrium (HWE) was assessed using the χ^2 test.

Assessment of the Effects of Interferon-γ (IFN-γ) and Cigarette Smoke Extract (CSE) on TNF-α Responses Elicited by Endotoxin from THP-1 Cells

Tumor necrosis factor-α responses of the THP-1 human monocytic cell line were measured as previously described in the model system to assess the effects of surrogates for infection (IFN-γ) and exposure to CSE on cells with a uniform genetic background (19).

Assessment of the Effects of IFN-γ and CSE on TNF-α Responses from Human Peripheral Blood Monocytic Cells

Collection, isolation, and storage of human peripheral blood monocytic cells (PBMC) have been described previously. Buffy coats from 14 male and 14 female donors, aged 20–55 years, were purchased from the Australian red cross blood service (ARCBS) (Sydney, Australia). Ethical permission was obtained from University of Newcastle Human Research Ethics Committee (H-229–0606) and ARCBS Ethics Committee (07-11NSW-07) for the purchase and use of human buffy coats for the purposes of the study. PBMC were collected from each donor for *in vitro* cytokine stimulation assays and plasma was collected for the assessment of cotinine for evidence of exposure to cigarette smoke, a confounding variable for altered cytokine responses. *TNF* G-308A genotype was determined as described above. Donors with detectable levels of cotinine were excluded from the analysis. Only ARCBS donor samples that were cleared for infectious agents were received. Leukocytes were collected and stored as described previously (15).

In our initial studies, TNF-α responses were examined with blood samples (10–20 ml) collected from British parents of SIDS infants ($n = 34$) and a comparison group, individuals unrelated

to the families and who had no family history of SIDS ($n = 59$). DNA was extracted from the leukocytes and screened for the *TNF* G-308A alleles as described above. The leukocytes were assessed for inflammatory responses to TSST-1 and LPS as described previously. All samples were coded and tested without knowing the sex or smoking status of the donors. Leukocytes (1×10^6 cells ml^{-1}) were stimulated *in vitro* with 0.01 μg ml^{-1} or 1 μg ml^{-1} *Escherichia coli* LPS or 0.5 μg ml^{-1} TSST-1 (Sigma, Poole, Dorset, UK) for 24 h. Cell culture conditions have been described previously (13).

Quantitative Assessment of TNF-α

Production of biologically active TNF-α was assessed by bioassay with L929 cells as described previously. The results were expressed as international units derived from the standard curves obtained using a recombinant human TNF-α standard.

Statistical Methods

Student's *t*-test was used on $\log_{10}$ -transformed data to assess differences in TNF-α responses for the various experimental conditions tested. The significance level for all tests was set at $p < 0.05$.

Results

The distributions of genotypes among the different groups assessed are summarized in **Table 1**. Distributions were in HWE for each of the groups assessed.

Distribution of *TNF* G-308A among SIDS Infants

The majority of each SIDS group possessed the GG genotype; however, Australian SIDS infants had a significant increase in proportion with the AA genotype (6/25, 24%) compared to the Hungarian (0/18, 0%) ($p = 0.09$) and German (0/46, 0%) ($p < 0.01$) SIDS infants.

The distribution of allele frequencies for the Australian control population differed significantly from that observed for Australian SIDS infants ($p = 0.02$). Only 3/62 (5%) of controls had the AA genotype compared with 24% of SIDS infants. No significant differences were detected between the distribution of *TNF* G-308A genotype for the combined SIDS infant group compared to the Caucasian controls ($p = 0.41$).

Assessment of *TNF* G-308A among Parents of SIDS Infants

The distribution of *TNF* G-308A genotype among parents of SIDS infants was not significantly different compared with their respective control populations. Parents of SIDS infants recruited from Australia showed an increased proportion of individuals with the GA genotype compared to their matched Australian controls but this was not significant ($p = 0.22$). Parents of SIDS infants recruited from Britain had a genotype distribution similar to their control population; the majority of individuals had the GA genotype ($p = 0.89$).

Distribution of *TNF* G-308A in Different Ethnic Groups

The distribution of *TNF* G-308A varied significantly among individuals from different ethnic groups. The majority of both British and Australian control populations possessed the GG genotype with fewer than 5% of individuals possessing the AA genotype.

As there were no differences between the allele frequencies for British and Australian control populations, these data were combined for further comparison with the Bangladeshi and Aboriginal Australian populations. The distribution of allele frequencies differed significantly between the Caucasian group and both Bangladeshis ($p = 0.01$) and Aboriginal Australians ($p < 0.01$). More than 90% of the Bangladeshi and Aboriginal Australian populations possessed the GG genotype, and 0% had the AA genotype.

TABLE 1 | *TNF* G-308A allele frequency distribution in the study populations.

Ethnicity	Group		Allele frequency (%)			Sample size (*n*)	*p* value (*p*)
			GG	GA	AA		
British	SIDS	Parents	65	35	0	34	0.89
British	Control	Parents	66	32	2	56	
Australian	SIDS	Parents	57	40	3	60	0.22
Australian	Control	Parents	69	26	5	62	
Australian	SIDS	Infants	48	28	24	25	0.03[a]
Hungarian	SIDS	Infants	61	39	0	18	0.05[b] 0.09[c]
German	SIDS	Infants	85	15	0	46	<0.01[d]
Combined	SIDS	Infants	70	24	7	89	0.41[e]
Bangladeshi	Control		94	6	0	32	0.01[f]
Aboriginal Australian	Control		99	1	0	117	<0.01[g] 0.12[h]
Caucasian	Control		68	29	3	118	

[a] *Australian SIDS infants vs. Australian control parents.*
[b] *Hungarian SIDS infants vs. German SIDS infants.*
[c] *Hungarian SIDS infants vs. Australian SIDS infants.*
[d] *German SIDS infants vs. Australian SIDS infants.*
[e] *Combined SIDS infants vs. Caucasian control.*
[f] *Bangladeshi control vs. Caucasian control.*
[g] *Aboriginal Australian control vs. Caucasian control.*
[h] *Aboriginal Australian control vs. Bangladeshi control.*

Effects of INF-γ and CSE on TNF-α Responses of THP-1 Cells to LPS

Unstimulated THP-1 cells had no measurable TNF-α in response to INF-γ at 1 ng ml^{-1} (low) or 10 ng ml^{-1} (high) concentrations. Priming of THP-1 cells with INF-γ resulted in dose-dependent enhancement of TNF-α responses to LPS (50 ng ml^{-1}) (**Figure 1A**). Neither low (L) (0.0001 cigarette per milliliter) nor high (H) (0.001 cigarette per milliliter) concentrations of CSE elicited measurable TNF-α. There was a dose-dependent but non-significant reduction in TNF-α responses to LPS in the presence of low dose of CSE (**Figure 1B**). In the presence of the high dose of CSE and either high or low concentrations of IFN-γ, TNF-α responses were significantly enhanced compared to stimulation with LPS alone (**Figure 1C**). The IFN-γ-enhanced TNF-α responses were not reduced by the CSE exposure.

Effects of INF-γ and CSE on TNF-α Responses of Human PBMC to LPS

Peripheral blood monocytic cells from 28 blood donors who had no evidence of cigarette smoke exposure or infection were used for assessment of the effects of CSE and INF-γ on TNF-α responses to LPS in relation to genotype. Baseline levels of TNF-α responses elicited by the individual components and combinations of components were approximately twice those elicited from THP-1 cells (**Figure 2A**). INF-γ priming significantly enhanced TNF-α responses ($p < 0.0001$). As observed with THP-1 cells, CSE did not elicit TNF-α from PBMC. In contrast to the results obtained with THP-1 cells, the high dose of CSE significantly reduced TNF-α responses to LPS ($p < 0.0001$) (**Figure 2B**). The presence of both INF-γ and CSE resulted in enhanced TNF-α responses compared with LPS alone; however, the response was lower if pre-treated with CSE and INF-γ than if pre-treated treated with INF-γ alone ($p < 0.0001$). (**Figure 2C**).

When assessed by gender of the donor, there were no significant differences between: TNF-α responses to LPS, INF-γ, or CSE; or combinations of LPS with CSE or LPS with INF-γ. There was a significant difference in TNF-α responses of cells from female donors (6510 pg ml^{-1}) compared with responses from cells of male donors (4621 pg ml^{-1}) if both INF-γ and CSE were used to pre-treat cells prior to exposure to endotoxin ($p = 0.014$). There were no significant differences in TNF-α responses associated with genotypes GG ($n = 15$), GA ($n = 10$) or AA ($n = 3$) under any of the conditions tested. For donors with the GG genotype, there were higher responses to LPS from cells of female donors ($n = 9$) (1674 pg ml^{-1}) than from cells of male donors ($n = 6$) (989 pg ml^{-1}) ($p = 0.03$). While the responses from cells of females with the GA genotype ($n = 2$) were approximately twice that of those from cells of male donors ($n = 8$), the differences were not significant ($p > 0.05$). There were only three donors with the AA genotype and all were female.

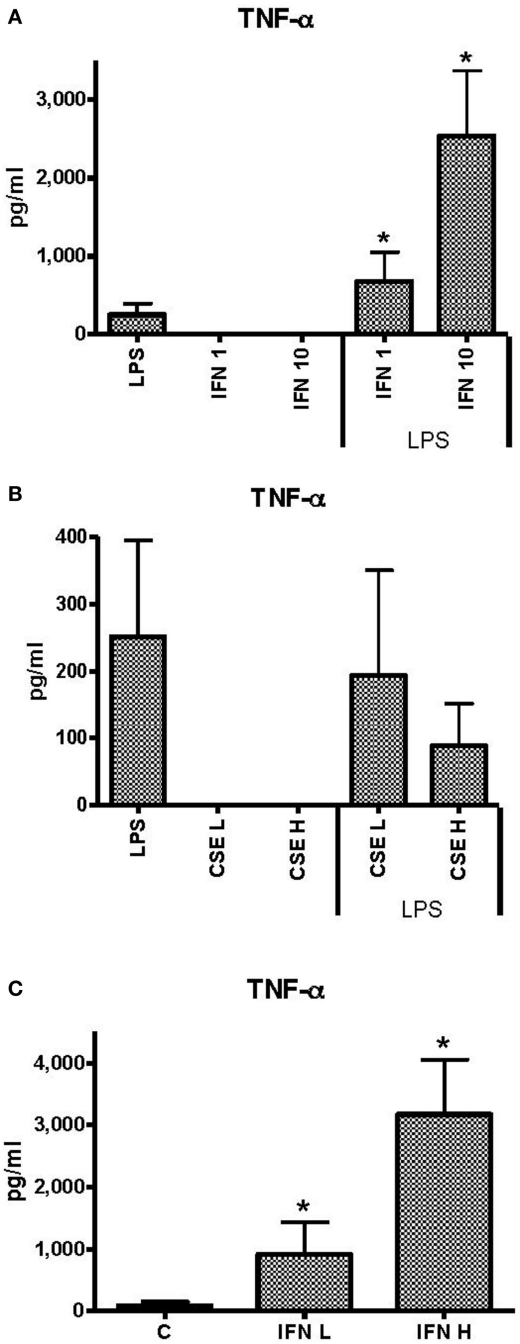

FIGURE 1 | Effects of IFN-γ and CSE on TNF-α responses of THP-1 cells to LPS (50 ng ml^{-1}). **(A)** TNF-α responses of THP-1 cells to LPS (50 ng ml^{-1}) and IFN-γ at 1 ng ml^{-1} (IFN 1) or 10 ng ml^{-1} (IFN 10) (*$p < 0.05$). **(B)** TNF-α responses of THP-1 cells to LPS (50 ng ml^{-1}) and CSE at low level (CSE L = 0.0001 cig ml^{-1}) and high level (CSE H = 0.001 cig ml^{-1}). **(C)** TNF-α responses of THP-1 cell to LPS (50 ng ml^{-1}) and CSE (0.001 cig ml^{-1}) and IFN-γ at low level (IFN L = 1 ng ml^{-1}) and high level (IFN H = 10 ng ml^{-1}) (*$p < 0.05$).

Effect of Smoking, Gender, and *TNF* G-308A on TNF-α Responses to LPS or TSST-1

This experiment utilized cells from the UK donors. There were no significant differences in the median TNF-α response to TSST-1 or LPS associated with different genotypes of *TNF* G-308A for smokers ($n = 38$) or non-smokers ($n = 60$). For the GG and GA genotypes, the geometric mean TNF-α responses were below 10 IU ml^{-1}. For the three donors with the AA genotype, there was one smoker and two non-smokers. The responses of the smoker donor with the AA genotype were similar to those of the GG

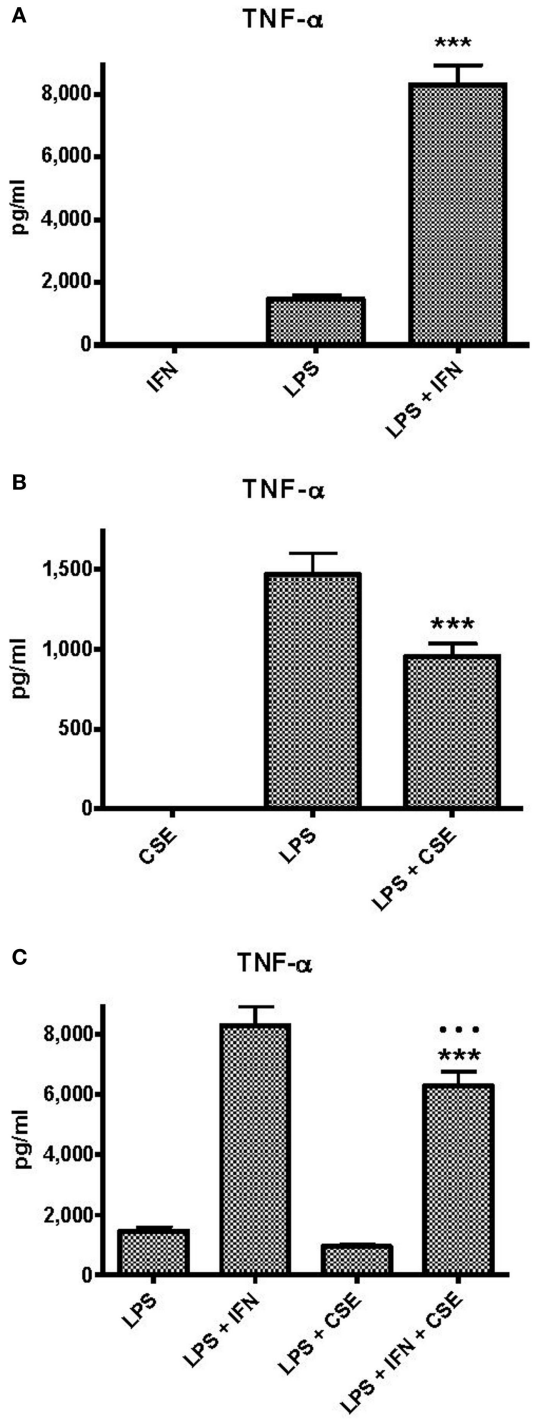

FIGURE 2 | Effects of IFN-γ and CSE on TNF-α responses of PBMC to LPS (50 ng ml^{-1}). **(A)** Effect of IFN-γ (10 ng ml^{-1}) pre-treatment on LPS stimulation of TNF-α from PBMC ($n = 28$) (***$=p < 0.0001$). **(B)** The effect of CSE (0.001 cig ml^{-1}) pre-treatment on LPS (50 ng ml^{-1}) stimulation of TNF-α from PBMC ($n = 28$) (***$=p < 0.0001$). **(C)** The effect of CSE (0.001 cig ml^{-1}) and IFN-γ (10 ng ml^{-1}) pre-treatment on LPS stimulation of TNF-α from PBMC ($n = 28$) (***$=p < 0.0001$).

and GA donors (<10 IU ml^{-1}). The response of the two donors who were not smokers was nearly 20 IU ml^{-1} for responses to TSST-1 and over 50 IU ml^{-1} for responses to the higher dose of LPS. There were not enough donors with the AA genotype for statistical analyses.

Discussion

In this study, we found an association with the *TNF* G-308A genotype AA in Australian SIDS infants. Given the rarity of the *TNF* G-308A A allele in the Caucasian population, it was an unusual observation that 24% of the Australian SIDS infants tested in this study were homozygous. The number of infants assessed in this study was small; therefore, data should be interpreted with caution. In the Norwegian study, 3% of SIDS infants, 5% of borderline SIDS, and 3% of infants who died of infection had the AA genotype; while none of the controls had this SNP genotype (11).

While the distribution of *TNF* G-308A genotype in the Indigenous Australian population differed significantly from that in the Caucasian population, the groups with high (Indigenous Australian) or low (UK Bangladeshi) incidences of SIDS had similar genetic profiles.

If the finding of a significant increase in the AA genotype associated with high TNF-α reflects a risk factor for SIDS, the findings need to be assessed in relation to the various hypotheses proposed to explain the physiological events leading to SIDS.

Long QT interval is reported to be an important risk factor for SIDS based on a prospective study of a large group of infants (20). The resident myocardial macrophages and mast cells and cardiomyocytes synthesize TNF-α. Arrhythmia has been reported as a side effect of treatment of patients with metastatic cancer with TNF-α, IL-2, and IFN-γ (21, 22). Negative ionotropic and arrhythmogenic effects were observed in myocytes cultured in IL-1, IL-2, IL-3, and TNF-α (23).

Dysregulation of glucose metabolism was proposed as one mechanism triggering the physiological events leading to SIDS (24). Acute hypoglycemia was associated with deranged cytokine levels. Hypoglycemia induced in rats with TNF-α without changes in the insulin levels was ameliorated by corticosteroid therapy (25). Hypoglycemia in an elderly patient with non-Hodgkin's lymphoma was associated with normal insulin and insulin-like hormone levels but high TNF-α levels. Glucose homeostasis was normalized after lowering TNF-α by cytoreductive therapy (26).

The A allele has been associated with increased production of TNF-α (27, 28). The cytokine response, however, is multi-redundant and pleiotropic; therefore, inactivation or over production of a particular cytokine might not have an effect on its own. When combined with other imbalances in the cytokine cascade, real physiological differences could be observed. It is, therefore, important to interpret cytokine and cytokine SNP data with caution and to consider the effects of other genetic, developmental, and environmental influences on these responses.

Despite finding interactions with smoking and cytokine gene polymorphisms in our previous studies (13), this study found no significant effects of gender, active smoking, or *TNF* G-308A genotype on TNF-α responses to TSST-1 or LPS in the different populations. Although the responses of the non-smokers with the AA genotype were higher than those observed for the other genotypes, there were too few donors with this genotype for

statistical analyses. These studies need to be repeated with more samples from individuals with the rare genotype.

In the experiments with cells from healthy Australian blood donors which were controlled for exposure to cigarette smoke and infection, females generally had higher TNF-α responses than males (15). When assessed by gender, females with the AA genotype had the lowest responses to the different conditions tested. This is in contrast to the results with cells from UK donors in which the rare AA genotype had the highest TNF-α responses to either TSST-1 or LPS. There are several factors that could contribute to the differences. In the study with Australian donors, TNF-α was assessed by Bio-Rad 6-plex assays and the Luminex 200 analyzer; this method detects total TNF-α. TNF-α responses from cells of the UK donors were tested for cytotoxicity for the L929 cell line which measures the biologically active TNF-α. The numbers of Australian donors tested were also smaller than those in the UK study. The UK samples were not assessed for level of exposure to cigarette smoke or concurrent viral infections which we have demonstrated can significantly alter cytokine responses including TNF-α. For the donors in the UK study, exposure to cigarette smoke was assessed only by self-reported smoking.

In conclusion, the *TNF* G-308A genotype results indicated that for the Australian population, infants with the AA genotype might be at increased risk of SIDS. The study needs to be expanded and investigations need to include thorough investigation for microorganisms and their toxins as well as investigation of the genetics and cytokine responses. While the effects of genotype on TNF-α responses remain unclear in the models tested, the effects of INF-γ are unequivocal for both THP-1 cells and PBMC. The results indicate that virus infection might enhance TNF-α responses to minor bacterial infection. While exposure to cigarette smoke is a risk factor for SIDS, it does not appear to enhance TNF-α responses to LPS. For the PBMC studies, CSE reduced significantly the enhanced responses of IFN-γ primed cells to LPS.

Author Contributions

Each of the authors made substantial contributions to the conception, design, analyses, and interpretations of the work. They assisted in preparing the article, critically assessed the final version and agree to be accountable for the accuracy and integrity of the work.

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Cytokine levels in late pregnancy: are female infants better protected against inflammation?

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Inflammatory responses have been implicated in several forms of infant deaths (sudden expected deaths and stillbirths) and the initiation of pre-term births. In this study, we examined matched samples of term maternal blood, cord blood, and amniotic fluid obtained from 24 elective cesarean deliveries for both pro- and anti-inflammatory cytokines thought to be important in maintaining a balanced response leading to successful pregnancy outcome. These included interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-10, and IL-1 receptor antagonist (IL-1ra). Amniotic fluid levels for each of the cytokines examined were significantly higher than those for cord blood or maternal plasma. While pro-inflammatory cytokines were higher in amniotic fluid associated with male fetuses compared with females, the major significant difference was higher levels of IL-1ra in amniotic fluid associated with female fetuses. Our study supports similar findings for cytokines during mid-trimester, which noted that amniotic fluid levels were higher than those in maternal blood. Our study suggests that maternal decidua secretes additional IL-ra in the presence of a female conceptus which improves the likelihood of a good outcome compared to pregnancies with male fetuses.

Keywords: cytokines, amniotic fluid, maternal plasma, cord blood, third trimester

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Introduction

A balanced cytokine response is thought to be important in maintaining pregnancy (1). Our previous studies indicated that there were differences in inflammatory responses associated with sex and that testosterone levels present during critical development periods might influence pro-inflammatory responses to infection (2, 3). There is an excess of males among infants who die suddenly and unexpectedly (4, 5), stillbirths (6, 7), and pre-term births (8, 9). As inflammation has been implicated in each of these conditions, we examined cytokine levels in matched sets of samples of maternal plasma, cord blood, and amniotic fluid collected during elective term cesarean deliveries to determine: if in the third trimester, cytokine levels in maternal blood and amniotic fluid were higher than those reported for the second trimester (10); if there was a correlation between cytokine levels in maternal or cord blood or amniotic fluid samples; if there were differences in cytokine levels from samples associated with male and female fetuses.

Materials and Methods

Matched samples of maternal plasma, cord blood, and amniotic fluid were obtained from elective term cesarean section deliveries as part of a study approved by the Hunter New England Ethics Committee, Mapping the Progress of Human Parturition (02/06/12/3.13). Twenty-four sets of samples were examined, 12 from pregnancies with a female infant and 12 from pregnancies with a male infant. A Bio-Plex suspension array assay (Bio-Rad) was used to quantitate interleukin (IL)-1β, IL-6, IL-8, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and the anti-inflammatory mediators IL-10 and IL-1 receptor antagonist (IL-1ra) (11).

The samples were stored at −80°C. Specimens were thawed, vortexed, and a 200 µl aliquot filtered with a 0.2 µm centrifugal filter (Millipore). The filtrate was either refrozen at −80°C for later analysis or immediately diluted for cytokine assay in cold BioPlex diluent. Samples from maternal or cord blood were diluted one volume in two volumes and amniotic fluid was diluted one volume in three volumes of Bio-Plex sample diluent.

Results were expressed in pg ml^{-1}. Data were tabulated as median and ranges. Spearman's non-parametric correlations were used to determine relationships between variables. Student's *t*-test was used on log-transformed data to assess differences in cytokine levels between the three sources of samples and between male and female infants. Significance was set at 5%.

Results

The first objective of the study was to compare cytokine levels in maternal blood and amniotic fluid with data reported for an Australian population at mid-trimester (**Table 1**). In the current study, the median gestational age (GA) at time of sampling was 39.1 weeks. In many cord blood and maternal plasma samples in the current study, levels of IFN-γ, TNF-α, and IL-1β were below the lower limits of detection.

Median levels of maternal IL-1β, IFN-γ, TNF-α, and IL-ra were higher during the second trimester. IL-6, IL-8, and IL-10 were higher in the third trimester. For amniotic fluid, median levels for each of the cytokines assessed in this study were higher in the third trimester.

At mid-trimester, IFN-γ and TNF-α were found at higher values in maternal sera compared with amniotic fluid. In this study of samples collected in the third trimester, all the cytokines tested were found at significantly higher levels ($p < 0.00001$) in amniotic fluid compared with maternal plasma (**Table 2**).

In the mid-trimester samples, IL1-ra levels increased significantly with the age of the mother (10). In this study of samples obtained at term, the only correlation with maternal age was IL-8 ($r = 0.5789$, $n = 24$, $p = 0.0025$, $\alpha = 1$).

The second objective was to determine if there was a correlation between cytokine levels in maternal or cord blood or amniotic fluid samples obtained during the third trimester. Analyses among the three categories of samples were carried out on the pro-inflammatory IL-6 and IL-8 and the anti-inflammatory IL-10 and IL-1ra.

Levels of each of the cytokines tested were significantly higher in amniotic fluid compared with the matched maternal plasma or cord blood (**Table 2**). There were no differences in levels of cytokines between maternal plasma and cord blood.

There was a significant correlation between levels of IL-10 in maternal plasma and cord blood ($r = 0.8506$, $n = 24$, $p = 0.001$, $\alpha = 2$). There were no correlations between levels of any of the cytokines detected in cord blood and amniotic fluid. There was a significant correlation between levels of IL-1ra in maternal plasma and amniotic fluid ($r = 0.7293$, $n = 24$, $p = 0.001$, $\alpha = 2$).

The third objective was to determine if there were differences in cytokine levels in samples associated with male and female fetuses (**Table 3**). The levels of the anti-inflammatory IL-1ra were significantly higher in amniotic fluid from female infants ($p = 0.0009$). In amniotic fluid, median IL-1β levels were higher

TABLE 1 | Comparison of cytokine levels in maternal blood (a) and amniotic fluid (b) at trimester 2 (10) and trimester 3.

	Maternal blood	
	14–16 weeks *N* = 33	**37.6–40.3 weeks *N* = 24**
	Median (range) (pg ml^{-1}) *N* = 33	**Median (range) (pg ml^{-1}) *N* = 24**
IFN-γ	113 (34–431)	41 (<11–772)
IL-1β	7 (2–360)	<2.4 (<2.4–21)
IL-6	22 (6–2116)	59 (10–74)
IL-8	17 (3–98)	90 (36–3289)
TNF-α	53 (4–392)	16 (<5.4–32)
IL-10	3 (<1–1206)	11 (<1.8–160)
IL-1ra	284 (21–8620)	90 (<4–230)
	Amniotic fluid	
	14–16 weeks *N* = 100	**37.6–40.3 weeks *N* = 24**
	Median (range) (pg ml^{-1}) (*n* = 100)	**Median (range) (pg ml^{-1}) (*n* = 24)**
IFN-γ	22 (5–132)	1804 (15–14, 417)
IL-1β	1.5 (<1–5)	45 (<2.4–69)
IL-6	230 (28–2621)	2118 (646–12, 928)
IL-8	188 (29–1648)	2066 (578–7082)
TNF-α	18 (<1–153)	538 (24–3176)
IL-10	3 (<1–9)	73 (<1.8–303)
IL-1ra	758 (85–3833)	2357 (330–6295)

TABLE 2 | Comparison of median cytokine levels (pg ml^{-1}) in matched samples of maternal plasma, cord blood and amniotic fluid collected from elective cesarean deliveries ($n = 24$).

Cytokine	Median (range) (pg ml^{-1})		
	Maternal plasma	**Cord blood**	**Amniotic fluid**
Il-6	59 (10–74)	54 (12–3860)	2118 (646–12, 928)**
Il-8	90 (36–3289)	95 (23–333)	2066 (578–7082)**
IL-10	11 (<1.8–160)	12 (4–58)	73 (<1.8–303)**
IL1-ra	90 (<4–230)	85 (30–325)	2357 (330–6295)**

***Significant differences between amniotic fluid and cord blood and amniotic fluid and maternal plasma ($p < 0.00001$).*

TABLE 3 | Comparison by sex of the infant (males = 12, females = 12) of median cytokine levels in maternal plasma, cord blood, and amniotic fluid obtained during elective cesarean delivery.

Cytokines	Median (range) (pg ml^{-1})	
	Males	Females
Maternal plasma		
Il-6	64 (10–174)	46 (29–170)
Il-8	103 (88–3289)	78 (36–563)
IL-10	10 (<1–15)	13 (4–160)
IL1-ra	76 (4–220)	118 (83–230)
Cord blood		
Il-6*	70 (32–386)	39 (12–157)
Il-8	99 (59–197)	64 (23–333)
IL-10	10 (4–22)	15 (4–58)
IL1-ra	125 (30–325)	73 (30–293)
Amniotic fluid		
Il-6	1218 (398–12, 928)	2271 (292–10, 739)
Il-8	2403 (621–9179)	1794 (578–4186)
IL-10	112 (<1.8–303)	56 (9–226)
IL1-ra**	1839 (330–5065)	3909 (1786–8837)

*$p \leq 0.05$.
**$p = 0.0009$.

in samples from males (42 pg ml^{-1}) compared with those from females (28 pg ml^{-1}); however, there were two samples from females and three samples from males in which levels of IL-1β were below the lower limits of detection. There were negative correlations between IL-1β and IL-1ra: for all 24 samples, $r = -0.296$ but these were not significant. The negative correlation was more pronounced for samples from females ($r = -0.3457$) than those from males ($r = 0.1396$). For maternal plasma, there were no significant differences between the levels of cytokines and the sex of the infant. The only significant difference for cord blood was higher median levels of IL-6 for males (70 pg ml^{-1}) compared with those for females (39 pg ml^{-1}) ($p < 0.05$). IL-1β was measured; however, it was detectable in only four samples of maternal plasma and only one cord blood sample; therefore, it was not analyzed further.

Maternal plasma levels of IL-6, IL-8, IL-10, and IL-1ra were assessed in relation to body mass index (BMI) of the mother, birth weight of the infant and GA at birth. For all samples, neither IL-8 nor IL-6 was correlated with any of these three factors. IL-10 was not correlated with BMI or birth weight, but it increased with GA ($r = 0.3537$, $n = 24$, $p < 0.05$, $\alpha = 1$). IL-1ra was not correlated with birth weight or BMI, but it decreased as GA increased ($r = -0.5885$, $n = 24$, $p = 0.005$, $\alpha = 2$). For GA, there was a significant association between IL-8 levels with female infants ($r = 0.8860$, $n = 12$, $p < 0.001$, $\alpha = 2$), and for males, there was a significant negative correlation with IL-1ra levels ($r = -0.9115$, $n = 12$, $p < 0.001$, $\alpha = 2$).

For cord blood samples, there was no correlation between maternal BMI and any cytokine assessed. For birth weight, there were positive correlations with cord blood IL-6 levels for both females ($r = 0.666$, $n = 12$, $p < 0.05$, $\alpha = 2$) and males ($r = 0.5244$, $n = 12$, $p < 0.05$, $\alpha = 1$). For GA, there were no correlations with cytokine levels in cord blood for female fetuses. For males, there was a positive correlation between IL-6 levels and GA ($r = 0.6819$, $n = 12$, $p < 0.02$, $\alpha = 2$.

Although IL-1ra levels were significantly higher in amniotic fluid of female fetuses, when assessed by the sex of the fetus, the only significant correlation was between GA of male fetuses and IL-1ra ($r = -0.7225$, $n = 12$, $p = 0.01$, $\alpha = 1$). There was no correlation between birth weight or maternal BMI and levels of cytokines in amniotic fluid of either males or females.

In this study, IL-6 and IL-8 in amniotic fluid rose with GA of the infant; however, only the correlation with IL-6 levels was significant ($r = 0.3765$, $n = 24$, $p = 0.05$, $\alpha = 1$). Levels of the anti-inflammatory IL-10 and IL-1ra declined with GA, but these were not significant.

Discussion

The objectives of the study were to determine: if in the third trimester, cytokine levels in amniotic fluid and maternal plasma were higher than those reported for second trimester; if there was a correlation between cytokine levels in the samples obtained from mother and infant; if there were differences in cytokine levels associated with male and female fetuses.

To examine objective 1, data from Chow et al. (10) were compared with our findings as both studies used the same method for detection of cytokines in maternal blood and amniotic fluid. In both studies, IL-6, IL-8, and IL-1ra were detected in all samples of maternal blood. In the mid-trimester blood samples, IFN-γ, IL-1β, and TNF-α were within the range of detection. In the third trimester blood samples, the median levels of these cytokines were lower and many had undetectable levels. In both studies, IFN-γ, IL-6, IL-8, and IL-1ra were detected in all amniotic fluid samples. The median levels of all the cytokines tested (**Table 1**) were higher in amniotic fluid during the third trimester.

Median cytokine levels in 110 third trimester amniotic fluid samples from cesarean section deliveries not in labor (median age 39 weeks) were lower for IL-6 (764 pg ml^{-1}), IL-8 (629 pg ml^{-1}), IL-10 (6.6 pg ml^{-1}), and TNF-α (10.5 pg ml^{-1}) (12) than those reported in the present study. The only significant differences between these cytokine levels and those in samples taken during amniocentesis (median GA 17 weeks) were for IL-10, which was increased and TNF-α which was decreased (12). The higher levels reported in **Table 1** are unlikely to be associated with different GAs; the median GA for both studies was 39 weeks. Some of the differences might be attributed to methods for detection of the cytokines; the study by Weissenbacher et al. (12) used an enzyme linked immunosorbent assay.

There were few correlations of cytokine levels between samples from mother and infant. For the samples obtained at mid-trimester, macrophage inflammatory protein (MIP)-1β levels in maternal blood correlated with those in amniotic fluid (10). In this study, levels of IL-1ra in maternal plasma correlated with those in amniotic fluid and maternal IL-10 levels correlated with those in cord blood.

There were no sex-specific differences in cytokine levels in maternal plasma (**Table 3**). For cord blood, IL-6 was higher in males. For amniotic fluid, most of the pro-inflammatory cytokines, except IL-6, were higher in samples from male fetuses. The major significant difference for amniotic fluid samples was higher levels of IL-1ra in amniotic fluid of female fetuses.

The study of Chow et al. (10) found higher levels of IL-1ra in amniotic fluid compared with those reported by Bry et al. (13); at mid-trimester, there were no sex-specific differences. Our findings agree with those of Bry et al. for third trimester fetuses; the levels for IL-1ra were higher for female fetuses. The correlation that we observed between maternal plasma and amniotic fluid levels of IL-1ra suggests that the pregnant decidua secretes IL-1ra into both amniotic fluid and maternal blood. The relatively high levels of IL-1ra in amniotic fluid compared with maternal blood suggest that IL-1ra is preferentially secreted into the amniotic fluid or that clearance from this compartment is slower than from maternal plasma or a combination of both effects.

It has been proposed that pro-inflammatory cytokines (e.g., IL-6) are important in triggering birth (14, 15). For maternal plasma, both anti-inflammatory cytokines (IL-1ra and IL-10) declined with GA. In pregnancies, where the fetus was male the decrease in IL-1ra with GA was significant, and in male fetuses, there was an increase with GA in the pro-inflammatory cytokine IL-6 in their cord blood. In addition, if the fetus was male, there was a negative correlation between IL-1ra and GA in amniotic fluid. These findings indicate an increasing pro-inflammatory environment as term approaches; and it is most evident in pregnancies in which the fetus is male. IL-1ra levels were higher in the amniotic fluid of female fetuses, suggesting that if the fetus is female not only do levels of the anti-inflammatory cytokine IL-1ra remain constant but also they are significantly higher than those found in amniotic fluid when the fetus is male.

These findings provide preliminary normative data for our population for cytokine levels in late pregnancy and indicate that there are differences in inflammatory responses associated with male and female fetuses. They also indicate that sex of the fetus needs to be assessed in relation to the role of inflammatory responses in the outcome of pregnancy. More work is required to determine the interactions between factors reported to affect inflammatory responses such as higher testosterone levels associated with male fetuses (3, 16) and BMI, which is associated with increased in pro-inflammatory responses (17, 18). It is interesting to note that, in this study, we found that the baby's birth weight was positively correlated with cord blood levels of the pro-inflammatory cytokine, IL-6. Smoking is associated with decreased anti-inflammatory responses (19). The small number of smokers in the study did not allow us to examine the effect of this important risk factor.

Our results do indicate that female fetuses might be better able to deal with infection and inflammation *in utero* than males and mighty partly explain their lower incidences of pre-term births, stillbirths, and unexpected deaths in infancy. Our data also indicate that term is associated with an increase in inflammatory cytokines and a fall in anti-inflammatory cytokines that is consistent with inflammation playing a role in the onset of human labor. While preliminary, the results are relevant to the highly topical area of sex-specific changes in decidual immune function and the potential effects on both male and female fetuses.

Author Contributions

Each of the authors made substantial contributions to the conception, design, analyses, and interpretations of the work. They assisted in preparing the article, critically assessed the final version, and agree to be accountable for the accuracy and integrity of the work.

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Effects of maternal inflammation and exposure to cigarette smoke on birth weight and delivery of preterm babies in a cohort of Indigenous Australian women

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Sudden infant death syndrome (SIDS), neonatal deaths, and deaths from infection are higher among Indigenous Australians. This study aimed to determine the effects of inflammatory responses and exposure to cigarette smoke, two important factors associated with sudden death in infancy, on preterm birth, and birth weight in a cohort of Indigenous mothers. Indigenous Australian women ($n = 131$) were recruited as part of a longitudinal study while attending antenatal care clinics during pregnancy; blood samples were collected up to three times in pregnancy. Serum cotinine, indicating exposure to cigarette smoke, was detected in 50.4% of mothers. Compared with non-Indigenous women, the cohort had 10 times the prevalence of antibodies to *Helicobacter pylori* (33 vs. 3%). Levels of immunoglobulin G, antibodies to *H. pylori*, and C-reactive protein (CRP) were all inversely correlated with gestational age ($P < 0.05$). CRP levels were positively associated with maternal body mass index (BMI; $\rho = 0.449$, $P = 0.001$). The effects of cigarette smoke (cotinine) and inflammation (CRP) were assessed in relation to risk factors for SIDS: gestational age at delivery and birth weight. Serum cotinine levels were negatively associated with birth weight ($\rho = -0.37$, $P < 0.001$), this correlation held true for both male ($\rho = -0.39$, $P = 0.002$) and female ($\rho = -0.30$, $P = 0.017$) infants. Cotinine was negatively associated with gestational age at delivery ($\rho = -0.199$, $P = 0.023$). When assessed by fetal sex, this was significant only for males ($\rho = -0.327$, $P = 0.011$). CRP was negatively associated with gestational age at delivery for female infants ($\rho = -0.46$, $P < 0.001$). In contrast, maternal BMI was significantly correlated with birth weight. These data highlight the importance of putting programs in place to reduce cigarette smoke exposure in pregnancy and to treat women with chronic infections such as *H. pylori* to improve pregnancy outcomes and decrease risk factors for sudden death in infancy.

Keywords: Indigenous, pregnancy, SIDS, inflammation, smoking

INTRODUCTION

Among Indigenous Australians, there is a greater risk of sudden infant death syndrome (SIDS) compared with non-Indigenous Australians (1). This is perhaps not surprising as low birth weight and preterm birth, both risk factors for SIDS, are approximately twice as common among Indigenous Australians compared with non-Indigenous families (2). Additional perinatal risk factors for SIDS include poverty and exposure to cigarette smoke (3, 4), factors also more common among Indigenous Australians.

The burden of infection within the Indigenous population is high and accounts for 5% of the total burden of disease (5). Deaths due to infection are significantly more common among Indigenous Australian adults, particularly bacterial infections (6). Mild infection, either from a "cold," or an infection of a gastric nature, is often reported by parents of SIDS infants; and the risk factors for SIDS parallel those for infection (see Blackwell et al., this issue).

Infection stimulates the inflammatory response. The cytokines in turn stimulate the acute phase response and the endocrine production of the powerful anti-inflammatory hormone, cortisol. We have previously demonstrated that a central Australian Indigenous community had single nucleotide polymorphisms (SNPs) for cytokine genes that differed significantly from those of non-Indigenous Australians, and this SNP profile might contribute to powerful pro-inflammatory responses implicated in host

responses to infection (7). *In vitro* studies using peripheral blood mononuclear cells indicated that inflammatory responses elicited in response to the bacterial antigen lipopolysaccharide (LPS) were significantly affected by the sex of the donor, cytokine SNP profile, and surrogates for viral infection and cigarette smoke (8, 9).

Evidence of strong inflammatory responses among Indigenous Australians has also been reported. C-reactive protein (CRP) is an acute phase protein that is stimulated by infection and CRP levels rise during pregnancy (10). There are significant differences in levels of CRP associated with ethnic groups; black women in the United States had higher CRP levels than white women (7.68 vs. 2.59 mg/L) (11). Among Indigenous women in a remote community, median CRP levels were reported to be markedly higher (8 mg/L) (12) than those reported for American women in the Women's Health Study (1.5 mg/L) (13) and levels significantly increased with age.

One of the major risk factors for poor pregnancy outcome and infant deaths is maternal cigarette smoking during pregnancy. Due to the extended family arrangements in which many Indigenous women live, passive exposure to cigarette smoke is an important factor to consider. Considerable work has been done to reduce smoking in Indigenous communities with little improvement in many areas. It has been reported that many Indigenous women feel that smoking will reduce their perceived stress, and there is limited understanding of the impacts of smoking on the developing child in pregnancy (14).

In this study, we examined the effects of inflammatory responses and exposure to cigarette smoke among a cohort of Indigenous mothers participating in studies of pregnancy outcome on preterm birth and low birth weight, two important factors associated with sudden death in infancy. The study has been described previously (15). Our hypothesis was that risk factors we have found *in vitro* to contribute to the dysregulation of inflammatory responses (9, 16) might be more common in the Indigenous population, and that if present, the increase in inflammation might be associated with preterm birth and/or lower birth weight. Since our previous studies indicated that testosterone levels can affect pro-inflammatory responses (9), we assessed the effect of fetal sex on maternal inflammatory markers as fetal plasma testosterone is significantly higher in males (17). To examine this hypothesis, we addressed the following questions:

1. Is there evidence for increased inflammatory responses among pregnant Indigenous women?
2. Are inflammatory markers affected by risk factors associated with poor pregnancy outcome or sudden death in infancy?
3. Does evidence of inflammation or other risk factors for SIDS affect birth weight or gestational age at birth of male and female infants?

MATERIALS AND METHODS

STUDY DESIGN

This is a prospective longitudinal cohort study that was developed through a thorough community consultation and continues to be reviewed by the members of its Indigenous Steering committee. This study was approved by the Hunter New England Local Health District Human Research Ethics Committee (HNEHREC ref. no. 08/05/21/4.01, NSWHREC ref. no. 08/HNE/129), the University of Newcastle Human Research Ethics Committee (H-2009-0177), and the Aboriginal Health and Medical Research Council Ethics Committee (ref. no. 654/08).

The Indigenous women in this cohort have been recruited by Indigenous research assistants. Informed written consent was obtained in the presence of at least one family member. Recruitment of participants occurred when they were attending antenatal care during their pregnancy. The study design aimed to obtain one blood sample from each participant in each trimester, however sampling was opportunistic as there was a tendency for participants to attend clinics irregularly for care.

Some of the variables [immunoglobulin G (IgG) to *Helicobacter pylori* and cotinine] were compared with those obtained for samples from 150 healthy non-Indigenous, non-pregnant female blood donors. Approval was obtained from the Australian Red Cross Blood Sample (ARCBS) Ethics Committee (07-11NSW-07). Plasma was collected from buffy coats of 150 female blood donors for the assessment of cotinine, exposure to cigarette smoke, a confounding variable in assessment of inflammatory responses, and for evidence IgG antibodies to *H. pylori*.

SAMPLE COLLECTION

Venipuncture was conducted by a trained Indigenous Australian research assistant or a medical professional. Whole blood samples were analyzed immediately for white blood cell (WBC) count. Blood for biochemical analyses were collected into lithium-heparin or EDTA vacutainers as appropriate and placed on ice until serum or plasma was separated by centrifugation (3,500 rcf, 10 min) at 4°C. Samples were aliquoted and stored at −80°C until time of analysis of: CRP; total IgG, IgA, and IgM; IgG antibodies to *H. pylori*; cotinine; and cortisol.

SURVEYS

Participants were surveyed on their smoking habits and those of the people in their place of residence in an effort to determine if self-reporting of exposure to tobacco is reliable.

BIOLOGICAL ASSAYS

Parameters assessed in an earlier study of inflammatory responses among Indigenous Australians were used in this study (12): CRP; total immunoglobulins; WBC; IgG to *H. pylori*; exposure to cigarette smoke. In addition, the anti-inflammatory hormone cortisol was also assessed.

White blood cell count was measured from whole blood immediately following collection from participants using a Beckmann Coulter LH780 analyzer at Pathology North, Tamworth, NSW, Australia. CRP for each stored sample was determined using the Abbott Architect 8200 analyzer at Pathology North, Tamworth, NSW, Australia and total IgG, IgA, and IgM for each sample was determined by Hunter Area Pathology Service Immunology laboratory, John Hunter Hospital, New Lambton, NSW, Australia. Quantitative IgG antibodies specific for *H. pylori* were determined by adapting a commercially available quantitative enzyme linked immunosorbent assay (ELISA) kits (Bio-Rad Laboratories Inc., GAP™ IgG kit (Cat#4042002) based on previous studies relating to chronic infection and heart disease (18). Cortisol was analyzed

by Pathology North, Tamworth, NSW, Australia using the Abbott Architect 4100 analyzer.

Plasma from the samples from ARCBS donors and women in the study were assessed for exposure to cigarette smoke by a semi-quantitative commercial competitive enzyme immunoassay (EIA) kit according to manufacturer's instructions [Bio Quant Cotinine ELISA, CA. Catalog No. BQ 096D (96 wells)], as described previously (9).

STATISTICS

Data are expressed as medians and ranges and were analyzed using SPSS Version 21 (Chicago). Non-parametric tests were used to determine significant differences between trimesters. Spearman's non-parametric correlations were used to determine relationships between variables. Significance was set at 5%.

RESULTS

PARTICIPANT DEMOGRAPHICS

Data were obtained from 131 participants, of whom 31 had data collected at three or more visits during their pregnancies, 51 had data collected at two visits, and 49 had data collected at one visit only.

The median age of women in the Indigenous pregnancy cohort was 25 years (range of 13.8–40.9 years). The median body mass index (BMI) of the cohort was 30 (range of 15–52 kg/m^2). The number of past pregnancies ranged from 0 to 23 and the number of live children ranged from 0 to 9; 18% had a previous miscarriage (range 2–16); 2.5% reported a past stillbirth or sudden unexpected death in infancy (SUDI).

The median gestational age at delivery was 39.1 weeks (range 32–43 weeks). The median birth weight of the infants was 3180 g (range 910–5430 g).

INFLAMMATION

The markers for inflammation were assessed by trimester of pregnancy (**Table 1**). Cortisol and cotinine were also assessed by trimester.

Immunoglobulin G decreased significantly with trimester ($P = 0.003$); levels in the third trimester were significantly lower than trimester 1 or 2 ($P = 0.012$ and $P = 0.007$, respectively; **Table 1**). In contrast, IgM levels increased significantly with trimester ($P = 0.025$); levels in the first trimester were significantly lower than trimester 2 or 3 ($P = 0.014$ and $P = 0.009$, respectively). Levels of IgG, IgA, and IgM were higher among women carrying a female fetus, but the differences were not significant. Univariate analysis of IgG with trimester and sex as fixed factors showed that levels fell significantly with gestation and there was an interaction between sex and trimester ($P = 0.046$).

Compared with 150 non-Indigenous women of childbearing age, our cohort had 10 times the prevalence of detectable antibodies to *H. pylori* (33 vs. 3%). There was a significant inverse correlation between *H. pylori* antibodies and gestational age ($\rho = -0.202$, $P = 0.05$, $n = 94$).

C-reactive protein levels ranged from 0.2 to 109 mg/L. CRP levels were significantly lower in trimester 3 than in trimester 2 ($P = 0.031$) and CRP was inversely correlated with gestational age ($\rho = -0.153$, $P = 0.047$, $n = 170$). There was no association between CRP and cotinine. There was no significant change in WBC count during the three trimesters (**Table 1**).

While 47.6% of the women reported they were smokers, serum cotinine was detected in 50.4% of the mothers. The levels ranged from 0 to 440 ng/mL among women who were self-reported smokers. Cotinine was detected in two women who were self-reported non-smokers.

Table 1 | Markers for inflammation and smoking in each trimester of pregnancy in Indigenous Australian women.

	Trimester			Normal range[f]
	1	2	3	
IgG (g/L)	9.69 (7.66–14.6, $n = 9$)	9.17 (6.19–17, $n = 51$)	8.43 (3.38–240, $n = 90$)[d,e]	6.45–13.9[a]
IgA (g/L)	1.67 (0.81–2.21, $n = 9$)	1.62 (0.78–2.87, $n = 51$)	1.73 (0.52–862, $n = 90$)	0.80–4.12[a]
IgM (g/L)	0.70 (0.45–1.13, $n = 9$)	1.01 (0.32–3.4, $n = 51$)[d]	1.10 (0.32–3.4, $n = 89$)[d]	0.44–2.76[a]
H. pylori IgG (U/mL)	11.7 (8.6–28.4, $n = 9$)	9.45 (0.56–2.8, $n = 42$)	6.4 (2–82, $n = 43$)[d]	Negative <18, equivocal 18–20, positive >20
WBC (10^9/L)	9.9 (7–13, $n = 13$)	9.8 (3–17, $n = 55$)	10.5 (5–18, $n = 103$)	4.5–13.0[b], 4.0–11.0[c]
CRP (mg/L)	7.3 (0.5–26.3, $n = 11$)	7.5 (0.5–109, $n = 55$)	5.3 (0.2–92, $n = 104$)[e]	<5.0
Cortisol (nmol/L)	285 (198–500, $n = 8$)	360 (151–878, $n = 45$)	441.5 (13–733, $n = 90$)[d,e]	79–535
Cotinine (ng/mL)	36.9 (0–214, $n = 9$)	40 (0–440, $n = 49$)	0.3 (0–337, $n = 82$)	N/A

Data are presented as median (ranges, n).

[a] *> 16 years.*

[b] *16–21 years.*

[c] *> 21 years.*

[d] *Denotes significant difference from first trimester values.*

[e] *Denotes significant difference from second trimester values, $P < 0.05$.*

[f] *Normal non-pregnant ranges derived from Ref. (27).*

The levels of cortisol rose by trimester; levels were highest in the third trimester (**Table 1**; $P < 0.001$).

EFFECTS OF RISK FACTORS ON INFLAMMATORY MARKERS

Both WBC ($\rho = 0.283$, $P = 0.003$, $n = 108$) and IgG specific for *H. pylori* ($\rho = 0.216$, $P = 0.032$, $n = 99$) were associated with cotinine levels. In pregnancies carrying a male fetus, there was no association between IgG, IgA, or IgM levels and maternal BMI. There were, however, significant correlations in women carrying a female baby between maternal BMI and both IgG ($\rho = 0.59$, $P = 0.01$, $n = 18$) and IgA ($\rho = 0.58$, $P = 0.012$, $n = 18$). There was no association between any of the risk factors and the sex of the fetus with IgG antibodies to *H. pylori*. For WBC, a significant positive association with cotinine was found in mothers carrying either a male ($\rho = 0.32$, $P = 0.037$, $n = 44$) or female fetus ($\rho = 0.285$, $P = 0.05$, $n = 48$). Overall, CRP levels were significantly correlated with maternal BMI ($\rho = 0.449$, $P = 0.001$, $n = 56$, **Figure 1**); this was significant in mothers carrying male fetuses ($\rho = 0.55$, $P = 0.007$, $n = 23$) but not in mothers carrying female fetuses ($\rho = 0.442$, $P = 0.07$, $n = 18$). There was a negative correlation between IgG and cortisol levels ($\rho = -0.211$, $P = 0.027$, $n = 110$).

EFFECTS OF RISK FACTORS ON BIRTH WEIGHT

The median birth weight for female infants was 3127.5 g (range 1620–5430 g) and that for male infants 3090 g (range 910–5170 g). Birth weight was significantly correlated with gestational age at delivery ($\rho = 0.61$, $P < 0.001$, $n = 105$). For all infants, maternal serum cotinine levels were negatively correlated with birth weight ($\rho = -0.37$, $P < 0.001$, $n = 129$, **Figures 2A,B**): males ($\rho = -0.39$, $P = 0.002$, $n = 60$); females ($\rho = -0.303$, $P = 0.017$, $n = 62$). For females, birth weight was positively correlated with maternal cortisol levels ($\rho = 0.40$, $P = 0.013$, $n = 38$, **Figure 2D**). This was not observed for males. Maternal BMI was significantly correlated with birth weight for all infants ($\rho = 0.32$, $P = 0.005$, $n = 78$). When assessed by sex of the infant, this was significant only for males ($\rho = 0.5$, $P = 0.001$, $n = 38$, **Figure 2C**).

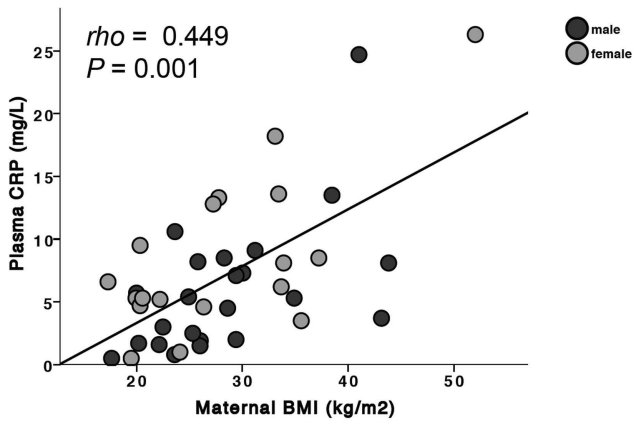

FIGURE 1 | Maternal BMI is positively associated with plasma CRP. When separated by fetal sex, this was significant in mothers carrying a male fetus (males: $\rho = 0.55$, $P = 0.007$) but did not reach statistical significance in mothers carrying a female fetus (females: $\rho = 0.442$, $P = 0.07$).

EFFECTS OF RISK FACTORS ON GESTATIONAL AGE AT BIRTH

For the combined male and female infants, maternal serum cotinine levels were negatively associated with gestational age at delivery ($\rho = -0.199$, $P = 0.023$, $n = 129$). When analyzed by sex of the fetus, this was significant only for males ($\rho = -0.327$, $P = 0.011$, $n = 60$, **Figure 3A**). CRP was negatively associated with gestational age only for female infants ($\rho = -0.46$, $P < 0.001$, $n = 64$, **Figure 3B**).

DISCUSSION

Our findings are discussed in relation to the hypothesis that Indigenous women have more powerful pro-inflammatory responses and that these might be enhanced by risk factors for SIDS, particularly smoking and high BMI. We chose our markers of inflammation based on results of a study of Indigenous Australians in a remote community (12) where for a cohort of 133 women, univariate analyses found CRP levels to be correlated with total IgG, IgA, and IgM, IgG antibodies to cytomegalovirus (CMV) and *H. pylori*, BMI, and cigarette smoking. Within this current study, we were unable to screen for IgG to CMV due to financial constraints. We assessed exposure to cigarette smoke by plasma cotinine, not just self-reported smoking in an effort to understand exposure to tobacco products in the home.

IS THERE EVIDENCE OF INCREASED INFLAMMATORY RESPONSES AMONG INDIGENOUS WOMEN IN THIS COHORT?

Normal levels of CRP are considered to be <1 mg/L (13). The current study found that women in the first and second trimesters had median CRP levels slightly above 7 mg/L and these fell to 5.3 mg/L in the third trimester. This is similar to levels reported in non-pregnant Indigenous women of a similar age (12). A study of CRP in American women found that at 26 weeks gestation, median CRP was 4.8 mg/L; however, there were differences associated with ethnic group. Black mothers had higher values (7.68 mg/L) than white mothers (2.59 mg/L) even after data were controlled for smoking and maternal weight (11). In contrast to our findings, they reported increasing CRP levels with increased gestational age but showed no association between CRP and smoking (11). Both McDonald et al. and Picklesimer et al. reported a positive correlation between BMI and CRP levels (11, 12), however non-pregnant Indigenous women had a lower median BMI (23.7 kg/m^2) than the pregnant women in our cohort (12).

Median IgG levels for women in our study (**Table 1**) were nearly half those reported for women in the remote community, 20.2 g/L (19.2–21.2). This might reflect a lower infection load compared with the women in the remote community, 68% of whom had evidence of IgG to *H. pylori* compared with a prevalence of 33% in our study. Total median IgA was also approximately three times higher in the remote community, 4.35 g/L (4.09–4.63) (12). Interestingly, there is a positive correlation between *H. pylori*-specific IgG and cotinine levels in our cohort. This association has been described previously (19) and indicates that exposure to cigarette smoke may contribute to the persistence of *H. pylori* infection. These data suggest that while the women in our cohort have similar levels of CRP compared to non-pregnant Indigenous women (12), this is likely to be related to increased obesity rather than a higher infection rate.

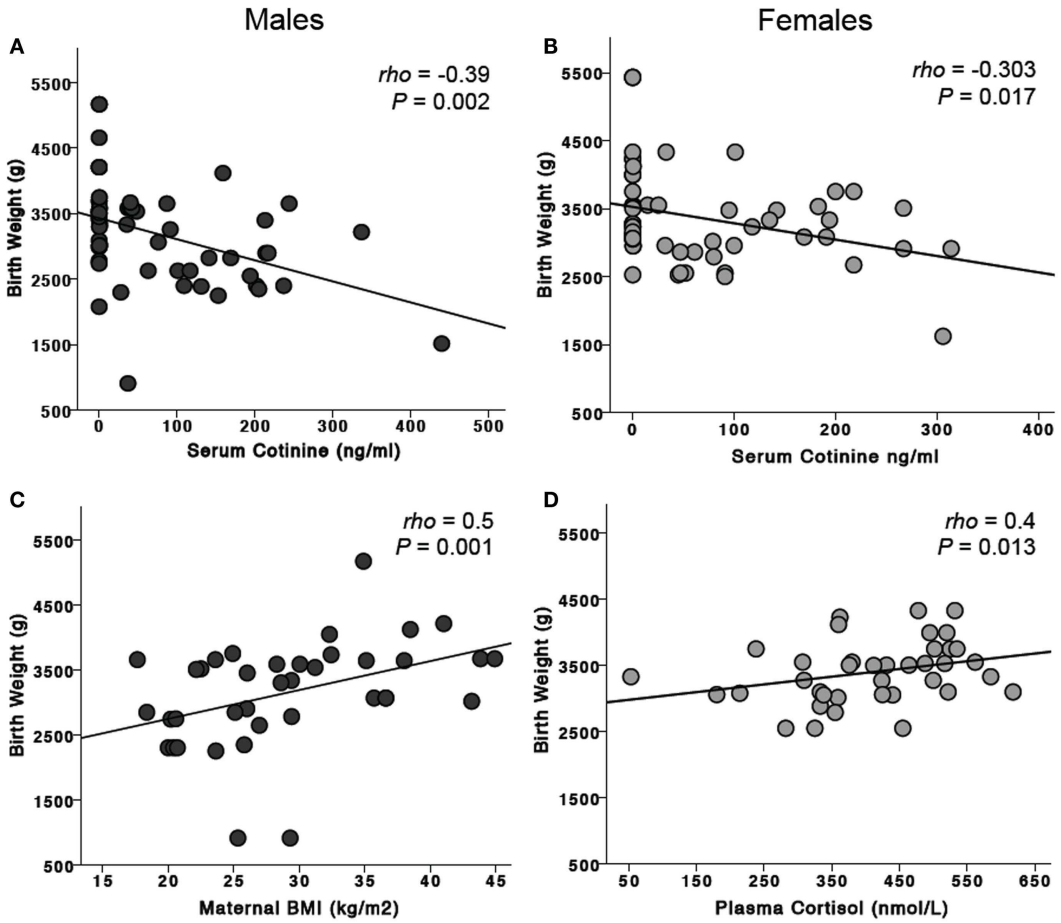

FIGURE 2 | Effects of risk factors on birth weight. Maternal serum cotinine levels were negatively associated with birth weight in mothers carrying both male **(A)** and female **(B)** fetuses. Maternal BMI was significantly associated with birth weight for males **(C)** but not females. In contrast, maternal plasma cortisol was positively associated with birth weight for females **(D)** but not males.

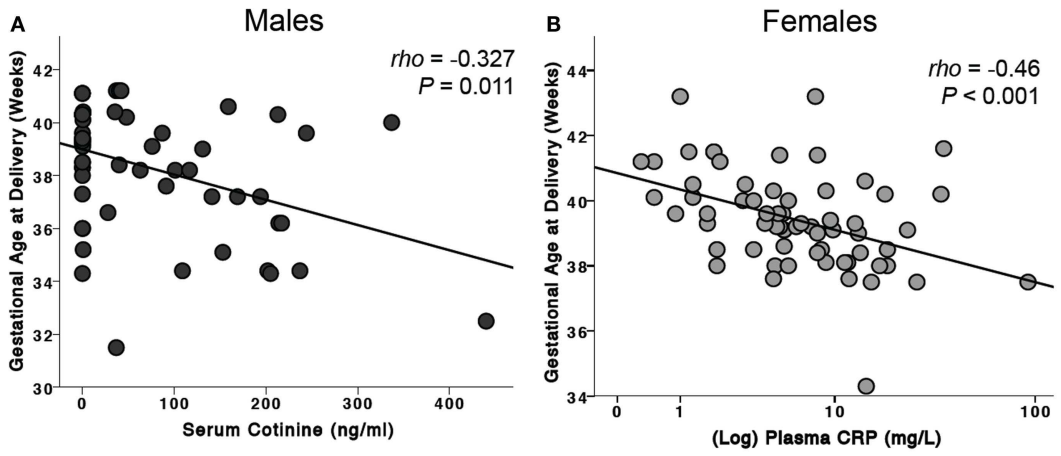

FIGURE 3 | Effects of risk factors on gestational age at birth. **(A)** For male infants, serum cotinine was negatively associated with gestational age at delivery. This association was not seen in female infants. **(B)** Maternal plasma CRP levels were negatively associated with gestational age of delivery of female infants. This observation was not found in males.

ARE INFLAMMATORY MARKERS AFFECTED BY RISK FACTORS ASSOCIATED WITH POOR PREGNANCY OUTCOME OR SUDDEN DEATH IN INFANCY?

Differences in inflammatory responses have been associated with the sex of the individual. In this study, we found that the sex of the fetus influences the levels of the mother's inflammatory markers – total IgG levels, WBC, and CRP. Maternal BMI affected both IgG and IgA levels if the mother carried a female fetus. WBC levels were correlated with exposure to cigarette smoke for both male and female fetuses but were more significant for males. For CRP levels, there was a correlation with BMI; this was significant for women carrying a male fetus and only marginally significant for women carrying a female fetus. These results reflect those we have previously observed *in vitro*; there are significant effects of sex on inflammatory responses, possibly associated with testosterone levels (9) and these interactions require further investigation.

DOES INFLAMMATION AFFECT BIRTH WEIGHT OR GESTATIONAL AGE OF MALE AND FEMALE INFANTS?

For all infants, maternal serum cotinine levels were negatively correlated with birth weight. For females, birth weight was correlated with maternal cortisol levels. BMI of the mother was significantly correlated with birth weight for combined male and female infants, but this was significant only in mothers carrying male fetuses.

For gestational age, there were negative correlations with cotinine levels, but these were significant only for males. There was a negative correlation with CRP levels but only in women carrying female fetuses.

The results indicate that male fetuses are more likely to be affected by risk factors that affect inflammatory responses that are associated with poor pregnancy outcome and sudden death in infancy – exposure to cigarette smoke and high maternal BMI. It is therefore not surprising that male infants are at greater risk of spontaneous preterm delivery (20–22), stillbirth (23, 24), and SIDS (25, 26).

CONCLUSION

There is evidence for higher levels of inflammation in pregnant Indigenous Australian women. This might reflect the high levels of obesity or the high levels of both acute and chronic infections in this population. Levels of CRP were negatively associated with gestational age in female infants suggesting that mothers with chronic inflammation who are carrying a female fetus may be more likely to deliver their babies early.

This study also highlights the adverse effects of exposure to cigarette smoke on birth weight in both male and female infants, which has been well documented. Importantly, we have also shown that males appear to be more affected by exposure to cigarette smoking than females, as cotinine is negatively associated with gestational age at delivery. Being born preterm and being male puts these infants at a significant disadvantage in terms of their neonatal outcomes and their risk of death in infancy.

AUTHOR CONTRIBUTIONS

KP, KR, EL, and CB all made substantial contributions to the conception, design, analyses, and interpretation of the work. They assisted in preparing the article, critically assessed the final version, and agree to be accountable for the accuracy and integrity of the work. SH and CB made contributions to the analyses and interpretation of cotinine and the immunoglobulin studies undertaken. LW recruited all participants within the Indigenous pregnancy cohort and made substantial contributions to the study design and assisted with editorial aspects of the paper. RS made substantial contributions to the conception, design, analyses and interpretation of the work, and assisted with the final editorial aspects of the paper.

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Animal models for assessment of infection and inflammation: contributions to elucidating the pathophysiology of sudden infant death syndrome

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Sudden infant death syndrome (SIDS) is still not well understood. It is defined as the sudden and unexpected death of an infant without a definitive cause. There are numerous hypotheses about the etiology of SIDS but the exact cause or causes have never been pinpointed. Examination of theoretical pathologies might only be possible in animal models. Development of these models requires consideration of the environmental and/or developmental risk factors often associated with SIDS, as they need to explain how the risk factors could contribute to the cause of death. These models were initially developed in common laboratory animals to test various hypotheses to explain these infant deaths – guinea pig, piglet, mouse, neonatal rabbit, and neonatal rat. Currently, there are growing numbers of researchers using genetically altered animals to examine specific areas of interest. This review describes the different systems and models developed to examine the diverse hypotheses for the cause of SIDS and their potential for defining a causal mechanism or mechanisms.

Keywords: anaphylaxis, development, hyperthermia, infection, inflammation, nicotine, serotonin, toxin

INTRODUCTION

Sudden infant death syndrome (SIDS) is the most common cause of infant mortality outside of the neonatal period in developed countries (1–3). It is a diagnosis made when every other possible cause can be excluded by the evaluation of all factors including an examination of the death scene, the review of the medical record, and a thorough autopsy (1, 4, 5). The incidence of SIDS is between 1 and 12 months of age with a peak between 2 and 4 months. Even though infants dying of SIDS appear normal and healthy, they often have had a recent mild viral illness that is not associated with the cause of death (1, 2, 6, 7).

Many of the risk factors associated with SIDS parallel those associated with susceptibility to infections. These include prone sleep position, maternal smoking, environmental tobacco smoke exposure, concurrent viral illness, ethnicity, gender, overheating, and drug or alcohol abuse during pregnancy (8–10). With the current decrease in SIDS due to the "Back to Sleep" campaign, the most significant risk factor continues to be maternal smoking during pregnancy (10–17).

Sudden infant death syndrome is most likely caused by several different mechanisms that trigger unexplained deaths. Animal models are important for evaluating the multiple hypotheses of causation and the relationships between: pathology related to sleep position; hyperthermia; hypoxia; reflux; anaphylaxis; infections; toxic exposures; substance abuse; and the role of neurotransmitters in regulation of basic physiologic functions. Animal models offer the ability to evaluate the interaction between different biologic systems in a single experiment.

This review will first outline the models used to assess the role of infection and inflammatory responses in these infant deaths. It will then review how the results of these studies might be applied to determine if inflammation could affect the physiological responses for other hypotheses proposed.

INFLAMMATION AND INFECTION

Several findings on autopsy of SIDS infants can be difficult to explain, particularly the finding of intrathoracic petechiae and liquid blood in the heart (18, 19). Evidence detected for inflammation has been summarized in the accompanying paper in this volume (Blackwell et al., this volume). These could be associated with efforts to overcome hypoxia, respiratory distress, suffocation, or resuscitation; however, they could also be due to coagulation problems associated with infection and inflammation (20, 21). There are often signs of inflammation and response to infection that are not due to the underlying, pre-existing symptoms. Thymic involution, is observed in more than half of SIDS cases and represents the response to severe stress (22). Thymic involution, intrathoracic petechiae, and liquid blood around the heart are also described in a developmental animal model of SIDS and are similar to the Rambaud results (23).

ANAPHYLAXIS

Anaphylaxis is a potentially lethal inflammatory response. It was one of the first hypotheses proposed to explain SIDS and was examined by successful development of an animal model (24). Studies were conducted to evaluate allergy associated with anaphylaxis in conscious guinea pigs that had been previously sensitized to cow's milk (**Table 1**). After challenge, these animals died quietly with some gasping and then appeared to fall asleep with the heart continuing to beat for a short time. These animals had two

findings commonly found on SIDS autopsy, fluid blood around the heart and an empty bladder. Additionally, there was significant histamine release (24). Buckley showed that beta tryptases were elevated in SIDS infants indicating possible mast cell degranulation prior to death (25). IgE levels were not affected but this is not a mandatory finding for anaphylaxis (25). Challenge from inhaled regurgitated milk in the lungs in a sensitized infant is a plausible scenario.

SYNERGY OF INFECTIOUS AGENTS

Assessment of the role of infectious agents and their toxins has employed several animal models. Each of these is considered below.

RABBIT

The response to intravenous injection of six common bacterial toxins was examined in 1–3 kg rabbits. The animals showed cardiac rate slowing, a drop in blood pressure, and apnea with sudden death. It was concluded that bacteria could produce toxins that cause inflammatory responses similar to those associated with endotoxin-induced shock (26) (**Table 1**). Catecholamine levels did not increase in these animals when the toxins were administered via the gastrointestinal tract; however, increasing doses administered intravenously demonstrated a dose-related increase in catecholamine levels and sudden death. A healthy gastrointestinal tract is not sensitive to these toxins (27).

CHICK EMBRYO

The lethality of toxins obtained from nasopharyngeal preparations from SIDS infants were tested individually and in combination in chick embryos. Enterobacterial and staphylococcal toxins alone were only lethal at high dilutions, however when combined, these same toxins were lethal at much lower concentrations. Both of these strains are found together more often in the nasopharynx of SIDS victims than in healthy infants (28) (**Table 1**). When nicotine was added at very low concentrations, it further potentiated the lethal action of these bacterial toxins (29).

WEANLING RATS

Weanling rats died rapidly without any symptoms prior to death following the injection of nasopharyngeal bacterial isolates obtained from SIDS. These animals had no signs of illness and negligible signs of inflammation in the heart, liver, and lungs (30) (**Table 1**). When *E. coli* and *S. aureus* were paired, the animals died more rapidly. Again this demonstrated the lethal synergy between different pathogens.

Table 1 | Animal models examining hypotheses to explain SIDS.

Hypothesis	Animal	Conclusion	Reference
Anaphylaxis	Guinea pig	Animals previously sensitized died quickly when exposed to cow's milk	(24)
Synergy of infectious agents	Rabbit	IV administration of different common bacterial toxins induced bradycardia, hypotension, and apnea in neonatal rabbits. Similar to endotoxin-induced shock	(26, 27)
	Chick embryo	Toxins from SIDS infants were lethal in combination. Nicotine potentiated the lethal effect	(28, 29)
	Weanling rats	Bacterial isolates from SIDS infants were lethal to rat pups when combined	(30)
	Neonatal rats	Pre-exposure to Influenza A virus caused a lethal response to a sub-lethal dose of endotoxin in rat pups on PN12	(31, 32)
	Mouse	Testing of toxigenic *E. coli* strains from SIDS infants increased mortality	(33)
	Mouse	Pre-exposure to gamma herpes virus and a sub-lethal dose of endotoxin increased fetal loss and reduced litter size	(34, 35)
Hyperthermia	Piglet	Head covering has its primary effect by increasing body temperature in piglets	(36–38)
	Piglet	Febrile piglets have a delayed response to airway obstruction	(39, 40)
Reflux and infection	Rabbit	Simulated reflux-induced apnea	(41)
	Piglet	Elevated body temperature decreased the protective respiratory response to chemoreflex	(42, 43)
Serotonin and inflammation	Serotonin-deficient mouse	Showed an inability to produce a protective autonomic response	(44)
	Mouse	Neonatal mice deficient in serotonin fail to auto-resuscitate during anoxia and die	(45, 46)
Nicotine and infection	Piglet	Nicotine and infection decreased the protective respiratory response to chemoreflex and hypoxia	(47, 48)
Nicotine and serotonin	Baboon	Prenatal nicotine exposure alters autonomic function and control of the heart via changes in the serotonin system	(49)
Nicotine and autonomic control	Rat	Prenatal nicotine exposure decreased auto-resuscitation in response to apnea. This response is directly associated with the effect of nicotine on the development of autonomic function	(50–57)
Development	Rat	On PN12, rat pups exhibit changes in their respiratory responses in the brain stem	(58–60)
	Rat	Lethal response to influenza A and sub-lethal endotoxin challenge in rat pups on PN12	(31, 32, 61)

NEONATAL RAT

Blood-Siegfried addressed susceptibility associated with developmental stage in relation to the timing of infectious insults. This model used a double insult with a non-lethal strain of influenza A virus and a sub-lethal dose of endotoxin. Animals were given an intranasal dose of the virus on postnatal (PN) day 10. When they were challenged with a sub-lethal dose of endotoxin 2 days after the viral exposure, 70% of the rat pups died within 8–10 h, quietly without significant symptoms. Animals displayed the characteristic intrathoracic petechiae, liquid blood around the heart, thymic involution, and other findings on pathology that have also been found in infants dying of SIDS (23). Older animals and younger animals did not die (31, 32). SIDS occurs between 2 and 4 months of age in human infants. The narrow window of lethality seen in this animal model on PN12 could be due to an increased susceptibility from normal developmental changes in the immune, endocrine system, or autonomic nervous system (31) (**Table 1**). These animals had an abnormal cytokine profile in response to that normally seen following endotoxin injection (62).

Pilot work suggests that rat pups exposed to nicotine *in utero* exhibited a similar pattern of mortality following endotoxin injection on PN11 or PN12. It was not necessary to pre-infect these animals with influenza. Prenatal nicotine exposure alone appears to be sufficient to alter the neonatal rat's physiology to make the animal susceptible to a sub-lethal dose of endotoxin. Work in this model is continuing.

MICE

Pregnant mice were exposed to *E. coli* in the drinking water. One strain of *E. coli* was toxigenic, obtained from SIDS infants; three other strains were from normal infants. Dams exposed to the toxigenic strain had smaller litter sizes and some runting of the pups. Mortality was 18% for the SIDS *E. coli* strain compared to 9% for non-SIDS isolates (33) (**Table 1**). In a different mouse model, gamma herpes virus plus a non-lethal dose of endotoxin was responsible for a reduction of litter size and fetal loss (34) (**Table 1**). These studies point to a double hit hypothesis for fetal and neonatal death. A single toxin or infectious insult might not be lethal on its own, but in the right combination, and at the right developmental stage, they trigger a lethal event. The multi-hit hypothesis also explains why the spiral to death can often not be stopped once it has started, even with exhaustive medical care. The process begins long before it is noticeable and treatment can be started.

In all of these models, an infectious insult was lethal to young animals. The number of infectious insults increased lethality with nicotine exposure also acting synergistically with the infectious agent. These data support the premise that maternal smoking, both prenatal and PN, might increase the lethality of a presumably non-lethal infection in a susceptible infant. Inflammatory mediators can affect many of the mechanisms proposed to explain SIDS. The following section assesses these models and comments on how inflammation might contribute to the lethal processes investigated.

RESPIRATION AND HYPERTHERMIA

Prone sleeping position plays a significant role in SIDS risk, not because of its effect on normal oxygen/carbon dioxide-exchange but rather on an increase in temperature. Elevated body temperature and increased toxin production by bacterial isolates in the nasal pharynx might be a contributing factor (33, 63, 64). Data suggest that prone position will exacerbate the consequences of a viral infection because it promotes an optimum temperature to increase the numbers and variety of bacterial species in upper respiratory secretions and stimulates bacterial toxin production (65).

It is well understood that infection affects the control of respiration and temperature. In studies of infants who died from SIDS while on heart monitors, the cardiac and respiratory tracings resembled those of infants with known infections rather than infants succumbing to asphyxia (66). Alteration of protective mechanisms, gasping, arousal, and efforts to restore normal blood pressure and heart rate are likely to be involved (67, 68). SIDS is more common in the winter months; therefore, these infants are usually well covered to keep them warm. Additionally, infants in a prone position tend to retain body temperature if overwrapped, even in cold weather, they risk becoming hyperthermic (69).

In a piglet model, Galland and colleagues (36, 37) tested the hypothesis that O_2 consumption could be altered in a cold climate when the face was cold and the body remained hot. They found that artificially induced hyperthermia increased REM sleep, a period associated with risk for SIDS (37) (**Table 1**). This study promoted the hypothesis that the primary effect on mortality was an increased body temperature rather than any changes in O_2 or CO_2 gas exchange (38, 70).

In a separate experiment, piglets developed tachycardia with a drop in blood pressure when heated. Their respiratory rate increased to compensate but they developed hypocapnic alkalosis and a metabolic acidosis. In addition, these animals had necropsy findings similar to that seen in SIDS cases, excessive lung hemorrhage, and alveolar edema (39) (**Table 1**). Febrile piglets were shown to have a delayed protective response to airway obstruction (40). Hyperthermia resulting from infection or prone position might trigger event leading to SIDS.

REFLUX AND INFECTION

Reflux during sleep has been proposed to have a negative effect on the human infant's ability of the human infant to auto-resuscitate. Simulated reflux in neonatal rabbits induced obstructive, central, and mixed apnea (41); however, the artificial instillation of a mildly acidic compound in a piglet model, stimulated normal protective responses in sedated and naturally sleeping animals (71, 72) (**Table 1**). Post-mortem lung changes in these piglets showed petechiae, characteristic of SIDS (73).

In a piglet model decerebrated, vagotomized animals were used to evaluate the effects of apnea and hyperthermia in sudden death. When body temperature was elevated, chemoreflex was prolonged (42) through a central nervous mechanism (43) (**Table 1**). Prenatal nicotine exposure decreased recovery from laryngeal chemoreflex in heated piglets (74). Again there was a connection with respiratory inhibition and hyperthermia.

Helicobacter pylori DNA and antigens have been found in post-mortem tissues of SIDS infants. A rat model has been used to determine if a fatal apnea could result from a response to gastroesophageal reflux (75, 76). The results were inconclusive that *H.*

pylori are a primary cause of SIDS; however, this model is consistent with infectious challenge (77–79). Recent work by Highet and colleagues on samples from SIDS infants show that the gut microbiome are significantly different than found in control infants and should be explored further as a cause of inflammation (80).

SEROTONIN, DEPRESSION, AND INFLAMMATION

The serotonergic system of the brain stem, an area that controls heart rate and breathing, is a primary focus for the role of the central nervous system in the factors underlying SIDS. The neurotransmitter serotonin appears to be very sensitive to inflammation; in particular, the inflammatory cytokines often found in autopsy in the central nervous system of SIDS infants (81–83). A specific relationship between increased inflammation and decreased serotonin output has been established in studies on depression (84); the use of anti-inflammatory medications improves serotonin function as it improves depression (85). If SIDS is associated with low levels of serotonin in important structures of the brain stem, it is reasonable to examine the hypothesis that inflammation due to infection might be involved.

Genetically altered animals have been used to explore specific pathology in the serotonin system. An over-expression of serotonin auto-receptors in this mouse model leads to death associated with a decrease in serotonin, drop in heart rate, hypothermia, and a failure to initiate protective autonomic responses (44) (**Table 1**). Neonatal mice deficient in brainstem serotonin have spontaneous bradycardia in room air and fail to auto-resuscitate during episodic anoxia, ultimately dying from an inability to appropriately increase heart rate (45, 46) (**Table 1**). Inflammatory cytokines decrease levels of serotonin in the brain stem and might lead to death because of an inability to respond appropriately to key triggers SIDS such as hyperthermia, hypoxia, low blood pressure or decreased heart rate.

NICOTINE

Smoking during pregnancy is a primary risk factor for SIDS (49, 86, 87). The risk is fairly low from environmental tobacco exposure (11, 13, 88–90); however, maternal smoking doubles the risk and that increases 3–4 times when the mother smokes more than 10 cigarettes per day (12, 86). Nicotine is considered a major neuroteratogen (91). Nicotine crosses the placenta during pregnancy and could explain why maternal smoking during pregnancy is more harmful on central respiratory mechanisms than PN exposure to environmental tobacco smoke (10, 13, 51). Prenatal nicotine exposure could result in an increase in susceptibility to other risk factors of SIDS in several ways that involve inflammation.

NICOTINE AND INFECTION

The synergy between infection and smoking has been examined in a piglet model. When animals are treated with nicotine and endotoxin then exposed to subglottic acidified saline solution are unable to auto-resuscitate and develop prolonged apnea. Nicotine and an inflammatory mediator interleukin-1β have a similar synergistic effect, decreasing the animal's ability to respond to apnea (47). These experiments demonstrate the synergistic connection between nicotine and inflammation and lethal apnea (92) (**Table 1**). While Froen and colleagues did not directly examine the role of infection, inflammatory mediators are often used as proxy for inflammation and it is highly likely that infection is involved.

In vitro studies on the effects of infection and risk factors such as cigarette smoke on inflammatory responses have identified that these factors can increase inflammation and enhance pro-inflammatory responses (93, 94), these effects need to be tested in animal models in which they also change the autonomic and serotonergic response to infection.

NICOTINE AND SEROTONIN

Prenatal exposure of animals to nicotine has been shown to alter the response of the serotonergic system in the newborn by down-regulating receptors important for normal serotonin function (49, 95). This in turn alters normal function and control of the heart through its action on the autonomic nervous system (49) (**Table 1**). Any process that challenges the cardiovascular system such as overheating, infection, toxins, and low blood sugar levels, increases SIDS risk (64, 96, 97). Nicotine exposure might increase the risk of SIDS by altering how the infant responds to these challenges.

NICOTINE AND AUTONOMIC CONTROL

Investigators have used the rat model to evaluate the effect of prenatal nicotine exposure on the heart (55), brain (54), and respiration (56, 57) (**Table 1**). Nicotine stimulates the nicotinic acetylcholine receptor and mimics the effects of acetylcholine (98). Chronic exposure of these receptors during critical prenatal development could explain the abnormalities of receptor expression and acetylcholinesterase activity observed in tissues from SIDS infants. These mechanisms are critical for normal autonomic control of the heart.

A strain of rabbits was developed to examine the effect of cardiac muscarinic receptors on autonomic tone. Similar to infants dying from SIDS, these rabbits had over-expression of muscarinic receptors that was most pronounced between the fifth and the seventh week of life, a time of increased mortality in the rabbits (99). Abnormal autonomic development from maternal smoking during pregnancy might result in infants being unable to arouse and respond to a physiologic insult such as a drop in blood pressure, hypoxia, infection, or overheating.

Mechanisms that regulate inflammation also affect vagal nerve activity through the release of acetylcholine. Data suggest an association between heart rate variability, a prime example of normal vagal function, and inflammation (100). Stress can precipitate both depression and promote inflammatory responses through its action on the sympathetic and parasympathetic nervous system (101). All of these processes are so intertwined that evaluating them as a single response negates the complex sets of interactions in the human body and encourages the use of animal models that can mimic interactions between systems.

DEVELOPMENTAL SUSCEPTIBILITY

One key factor in SIDS is the specific timing of susceptibility. By definition, SIDS infants are younger than 12 months of age with most SIDS occurring between 2 and 4 months. There is a peak around 3 months of age. Evaluation of a condition that occurs during such a specific time during the development of multiple interrelated physiologic systems can be quite difficult. Teasing

out the combined effects requires the ability to measure multiple interactions at the same time in many organ systems.

DEVELOPMENT OF INFLAMMATORY RESPONSES

The peak age at which SIDS occurs corresponds to a time when infants are dependent on innate responses to infection. A dysregulation of innate inflammatory responses and the developmental change that mature the infant could contribute to the physiological changes leading to these sudden deaths (31, 32, 94, 102, 103). The immune system is beginning to recognize a large number of foreign antigens and developing its own antibody responses while the protective effect of maternal antibodies is waning (104). Small changes in the development of immune responses can turn a minor illness into a lethal event (62). This could explain why breastfeeding is protective for SIDS because breast milk contains biologically active antibodies that reduce colonization by potentially pathogenic bacteria as well as glycoproteins that reduce attachment of organisms to epithelial cells via lectin like adhesins (105, 106).

Male infants succumb to SIDS slightly more frequently than females. Peripheral blood monocytic cells from adult male donors exposed to cigarette smoke extract produced lower levels of some inflammatory cytokines than those from adult female donors. There were significant correlations between levels of pro-inflammatory cytokines and testosterone, but only in females. Testosterone levels in females correspond to those among male infants in the age range at greatest risk of SIDS. There is potentially an effect between cortisol levels and the testosterone surge in male infants during the time of SIDS risk but this needs to be evaluated further (94).

The normalization of the adult day–night temperature pattern occurs around the time of highest SIDS risk (107). Both human infants (108, 109) and rat pups (110, 111) have a nadir of cortisol production during this time of development that overlaps a time when the immune system is responding to new infections and the corresponding cortisol system might not be able to regulate the response. The levels of cortisol suppress pro-inflammatory responses to bacterial antigens during the day, just before and after the switch to diurnal circadian rhythm. The night-time levels before the switch have the same effect; however night-time levels of cortisol after the switch are much lower and do not have a suppressive effect on the immune system. In fact, they can enhance pro-inflammatory responses [(106); Blackwell et al., this volume]. Developmental immaturity at this age could lead to an inability to produce a protective response to an infection.

DEVELOPMENT OF THE NEURAL SYSTEM

The maturity of the autonomic nervous system and baroreflex control of the heart in human infants correspond to times of autonomic imbalance (112). This imbalance is influenced by stage of sleep, head covering, temperature, body position, and nicotine exposure, all known risk factors for SIDS (113–116). In 2- to 4-month olds, there is a significant difference in cardiac response to a hypotensive event in quiet sleep. This is in contrast to that seen in adults and newborns where heart rate elevates and is maintained in an elevated stage for several minutes (117). Ledwidge et al. (118) describes a case where a 5-month-old infant died during a sleep study because the heart rate failed to increase in response to a head-up tilt (decrease in blood pressure), even though all other parameters of the sleep study were normal.

DEVELOPMENT AND RESPIRATION

Developmental changes in 12-day-old rats had an abrupt switch in brain stem expression of a receptor important for stimulating respiratory control. This period of imbalance is transitory but could leave the respiratory system vulnerable to insult such as infection that often causes apnea in infants (58–60, 119) (**Table 1**). These represent three very important areas of development that occur around the time of unexplained death that could be attributed to a SIDS-like event. This case supports the hypothesis that abnormal autonomic tone might contribute to SIDS deaths.

CONCLUSION

Any animal model used to mimic a human condition should reflect the pathophysiology and other parameters that are indicative of that condition. SIDS by definition is a sudden unexplained death that cannot be defined by symptoms or pathology commonly known to cause death. Because of this definition, there are very few clues to build a model on. The models presented here are an attempt to define possible clues on which to build a model with physical and epidemiologic findings of SIDS.

A developmental model like that described by Blood-Siegfried is critical to tease out the effect of inflammatory responses to infection on interactions among physiological systems and risk factors such as tobacco smoke (31, 32, 61, 120) (**Table 1**). This model not only causes pathological finding similar to those found on autopsy of SIDS infants but also reflects the narrow window of susceptibility not seen in many other models (23).

Further examination using this model could evaluate other possible mechanistic associations:

1) How does cigarette smoke extract affect 11- and 12-day-old rat pups?
2) How does the stress response as measured by catecholamine and corticosterone levels respond to the insult?
3) Do animals that are susceptible to death have specific changes in the circadian rhythms?
4) What is the normal development of autonomic tone and heart rate variability?

This developmental model allows the researcher to examine the effect of age within the context of the development of these systems and the interactions between systems.

We have only begun to tap into the multiple possibilities of connections involved in sudden and unexplained death. SIDS is a complex syndrome and the answer to its cause/s will require development of appropriate animal models to determine how all the parts fit together.

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The role of infection and inflammation in stillbirths: parallels with SIDS?

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It has been suggested that stillbirths are part of the spectrum of infant deaths that includes sudden infant death syndrome (SIDS). This paper examines the hypothesis that risk factors associated with stillbirths might contribute to dysregulation of inflammatory responses to infections that could trigger the physiological responses leading to fetal loss. These include genetic factors (ethnic group, sex), environmental (infection, cigarette smoke, obesity), and developmental (testosterone levels) factors. Interactions between the genetic, environmental, and developmental risk factors are also considered, e.g., the excess of male stillborn infants in relation to the effects of testosterone levels during development on pro-inflammatory responses. In contrast to SIDS, inflammatory responses of both mother and fetus need to be considered. Approaches for examining the hypothesis are proposed.

Keywords: stillbirth, sudden infant death syndrome, inflammation, infection, cigarette smoke, ethnicity, obesity

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Infection and Inflammation in Infant Deaths

There have been suggestions that stillbirths are part of the spectrum of infant deaths associated with sudden infant death syndrome (SIDS) based on epidemiological parallels (1). Some of the reported parallels included: ethnic background; maternal smoking; small for gestational age infants; evidence of infection/inflammation in mother and/or fetus. Infections have been implicated in the etiology of stillbirths in both developing and industrialized countries (2–4). As with SIDS and sudden unexpected deaths in infancy (SUDI), no single organism has been implicated (5–7). The common thread to be considered in this review is the inflammatory responses to infection and how the risk factors identified in epidemiological studies might affect these responses in both mother and infant. Based on our previous work on SIDS, our hypothesis is that genetic and environmental risk factors that result in dysregulation of inflammatory responses by mother and/or infant to infection could contribute to events leading to some unexplained stillbirths. **Table 1** lists the major risk factors for SIDS and for stillbirths and cites the supporting literature. In the following sections, the effects of genetic and environmental factors on inflammatory responses will be assessed.

Infection in Stillbirths

The incidence of stillbirths ranges from as few as 3/1000 births in developed countries to as many as 45/1000 in developing countries (25) where infection is more common.

Early studies implicated inflammation associated with infectious agents (26, 27). There are usually no overt signs of infection prior to fetal loss including: maternal fever or chills; abdominal discomfort; or fetal tachycardia. Ascending bacterial infection (before and after membrane rupture)

TABLE 1 | Comparison of risk factors for SIDS/SUDI and stillbirths.

SIDS/SUDI	Reference	Stillbirths	Reference
Ethnic group	(8–10)	Ethnic group	(11–13)
Male gender	(14, 15)	Male gender	(16, 17)
Cigarette smoke	(18)	Cigarette smoke	(12)
Infection	(5, 19)	Infection	(3, 4)
Prematurity	(20)	Prematurity	(12)
Small baby	(20)	Small baby	(12)
Overweight/maternal obesity	(21, 22)	Overweight/maternal obesity	(23, 24)

was identified as the most common infectious cause of stillbirth. Infection can also occur from hematogenous spread (4). The most common organisms involved were *Escherichia coli*, group B *Streptococcus pyogenes*, and *Ureaplasma urealyticum*. The two most common viral infections associated with stillbirths were parvovirus and Coxsackie virus (3, 26). A more recent study identified cytomegalovirus (CMV) in 15% of stillbirths (28). Serological studies have implicated *Chlamydia trachomatis* in a Scandinavian study of stillbirths (29). Although infection is considered a common cause of stillbirth, it is often hard to attribute this causally for a number of reasons. Several groups have studied the use of polymerase chain reaction (PCR) to identify specific viral and bacterial DNA and RNA and have found it to be more sensitive than routine microbiological methods in detecting evidence of infection in stillborn babies (28, 30).

Both invasive and toxigenic bacteria need to be considered as bacterial exotoxins or their cellular components can act as superantigens eliciting strong pro-inflammatory responses. A comprehensive review of the literature relating to stillbirth/intrauterine fetal death (IUFD) and infection suggested that between 10 and 25% of all cases of IUFD in developed countries were associated with infection (4). We identified pyrogenic toxins of *Staphylococcus aureus* in serum or tissues of over 50% of SIDS infants from five different countries (31) and toxins of enteric organisms have also been implicated (32); however, there has been no systematic assessment of material from stillbirths for presence of bacterial toxins.

The mechanisms proposed for the role of infection in stillbirths include (1) maternal illness resulting in fever, respiratory distress, or systemic responses to the infection; (2) infection of the placenta resulting in reduced fetoplacental blood flow; (3) direct infection of the fetus; (4) induction of pre-term labor (4). Inflammation might contribute to all of these, and the inflammatory response of the fetus also needs to be considered.

Inflammation and Infant Deaths

The inflammatory response is the major protective mechanism evolved to deal with pathogenic micro-organisms. The pro-inflammatory cytokines are involved in clearing the microorganism. The anti-inflammatory cytokines are involved in damping down the pro-inflammatory responses to prevent collateral damage of a too abundant response to infection; however, an innate tendency to enhanced anti-inflammatory signaling is thought to increase the risk of death through infection. Successful reproduction necessitates adequate immune tolerance to allow pregnancy to proceed without rejecting the fetus, half of whose antigens are from the father. Pro-inflammatory responses have been associated with increased resistance to infection and anti-inflammatory responses with increased fertility (33). Genetic and environmental factors that disturb the balance between pro- and anti-inflammatory cytokines might result in fetal damage. There is evidence that some ethnic groups at increased risk of infant deaths due to infection, SIDS or stillbirths (12, 23, 34). These include Indigenous communities in Canada and Australia and African-Americans (13, 23, 34). In these populations at higher risk of stillbirths, there is a general genetic predisposition to strong pro-inflammatory responses (35–40).

Women with low capacity to respond to vaginal infection through the production of pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and IL-8 might have a more permissive environment for pathogens to flourish and be at risk of ascending uterine infection and chorioamnionitis (41). Enhanced pro-inflammatory responses to vaginal infection or periodontal disease (42) are suggested to be detrimental to pregnancy and elevated levels of IL-6 have been found to be a predictor of pre-term labor (43, 44).

As with SIDS, histopathological changes in the placenta or fetus are not always consistent (45), and the presence of organisms does not always imply causation, although it is more likely if micro-organisms are found in fetal tissue compared with placenta or fetal membranes. Examination of placentas from live and stillborn infants found evidence of inflammation in 30.4% of stillbirths compared with 12% of controls. Inflammation was more common in placentas from early stillborn deliveries and also in early live births (46). Chorioamnionitis without fetal inflammatory responses was associated with stillbirth in early pre-term pregnancies (47).

It has been recommended that there is a need to assess the molecular evidence for inflammation in these deaths (48). In the case of SIDS, factors affecting the inflammatory responses of the infant need to be considered; for stillbirths, factors affecting the inflammatory responses of both mother and infant need to be considered. The methodology is available and preliminary studies on levels of cytokines in matched samples of maternal plasma, cord blood, and amniotic fluid from late pregnancy are reported in this issue (49). The levels of pro-inflammatory cytokines are significantly higher in the amniotic fluid compared with the levels in cord blood or maternal plasma (**Figure 1**).

Assessment of Inflammatory Responses in Relation to Risk Factors

Ethnic Group

The incidences of infant mortality due to infection, SIDS and stillbirths are higher among families of Indigenous groups (e.g., Aboriginal Australians and Native Americans) compared to families of European origins in the same countries (**Table 2**).

Ethnicity was a significant risk for stillbirth in the United Kingdom; those groups at increased risk included African, Afro-Caribbean, Indian, and Pakistani mothers. In other countries, Indigenous mothers have an increased risk for stillbirth. While these disparities have been ascribed primarily to socio-economic disadvantage, there is emerging evidence that genetic background

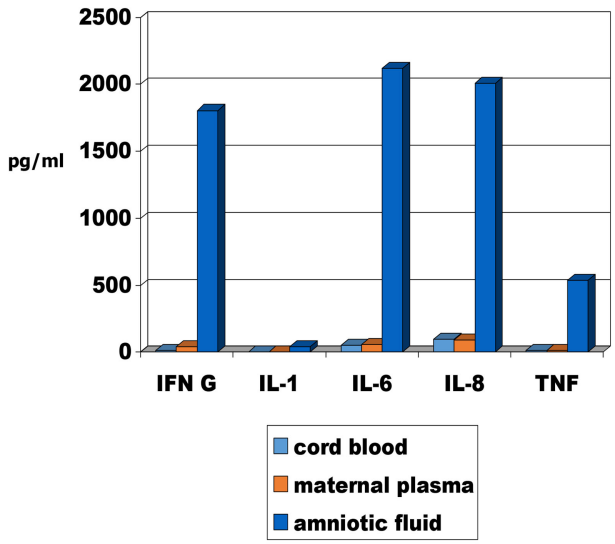

FIGURE 1 | Median pro-inflammatory cytokine levels in maternal plasma, cord blood and amniotic fluid of 24 mothers and infants obtained from elective Caeserean delivery [adapted from Burns et al. (49)].

TABLE 2 | Incidence of stillbirths among different ethnic groups.

Country	No/1000 births
United kingdom (12)	
European	3.2
Non-UK	4.0
African	7.4
Afro-Caribbean	6.7
Bangladeshi	4.2
Indian	
UK	3.9
Non-UK	6.4
Pakistani	
UK	4.1
Non-UK	6.9
Australia (11)	
Non-Indigenous	5.9
Indigenous	9.1
United states (13)	
Hispanic	5.44
Non-Hispanic white	4.75
Non-Hispanic black	11.13
Native American/Alaska Native	6.17

(40) and interactions between environmental factors such as cigarette smoke might contribute to susceptibility to, and severity of, inflammatory responses to infections (37, 50).

In relation to potential underlying factors affecting inflammatory responses and their role in stillbirths, it is important to note that cytokine gene polymorphisms associated with high-level responses of pro-inflammatory cytokines or low-level responses of anti-inflammatory cytokines such as IL-10 are predominant among some Indigenous groups, South Asians, and American Black populations (36, 40, 51). There are experimental and epidemiological studies indicating that genetic polymorphisms in the inflammatory response might contribute to poor pregnancy outcome; however, the results are inconsistent. Pre-term labor enhances the risk of stillbirth (4). Important risk factors include intrauterine infection/inflammation and social factors (stress, smoking, heavy work). The final common pathway appears to be activation of the inflammatory cascade. Bacterial infection and/or inflammation of the choriodecidual interface induces pro-inflammatory cytokine responses leading to neutrophil activation, synthesis, and release of prostaglandins causing uterine contractions and metalloproteinases weakening fetal membranes (52). Polymorphisms associated with increased production of pro-inflammatory and/or decreased production of anti-inflammatory cytokines have been implicated in pre-term birth. Those enhancing the magnitude or duration of the responses were associated with risk of pre-term birth (53, 54). *In vitro* studies with leukocytes from women with recurrent pregnancy loss found significantly higher levels of interferon-γ (IFN-γ) and a trend toward increased TNF-α production compared with women with no history of pregnancy loss. In relation to IL-6 and TGF-β, no significant differences were detected between the groups (55).

Modifiable Risk Factors and Inflammation

In relation to our previous studies on interactions between genetic and environmental factors on inflammatory responses, it is recommended that assessments of the genetic predisposition to inflammation need to control for environmental risk factors that alter cytokine responses to infection or toxins (37, 39). The major environmental factors to be considered in this review are co-infections, smoking, and obesity.

Infections

Virus pandemics have been associated with increased risk of pre-term labor and fetal loss (56, 57). There are a number of models that indicate that virus infections can potentiate the effects of bacterial toxins implicated in SIDS (58–60). There is also a mouse model that found that while an asymptomatic infection with the murine gamma herpes virus 68 did not disrupt pregnancy outcome, the infection could upregulate the pro-inflammatory responses to small quantities of endotoxin in both placenta and decidua, resulting in pre-term labor and fetal loss. Similar responses were observed for human primary trophoblast and trophoblast cell lines infected with this virus prior to exposure to endotoxin (61). The enhancement of pro-inflammatory responses to endotoxin was attributed to priming by IFN-γ and TNF-α responses to the virus infection. Additional evidence for the role of IFN-γ was provided by *in vitro* studies with human monocytic cells and the THP-1 cell line (50, 62) (Moscovis et al., this issue).

Chronic infections such as *Helicobacter pylori*, *Chlamydia pneumoniae*, and CMV can also significantly increase pro-inflammatory markers (63). *H. pylori* infection is significantly higher among mothers with small for gestational age infants (64). In a population in India, periodontal disease was associated with increased levels of C-reactive protein (CRP) and also with pre-term birth (65).

Cigarette Smoke

Both active smoking and passive exposure to cigarette smoke have been reported to enhance risk of stillbirth (12). Cigarette

smoke can influence infection and inflammation is several ways: (1) enhanced susceptibility to respiratory virus infection and subsequent enhanced colonization by potential bacterial pathogens; (2) increase in the numbers and species of respiratory bacteria due to enhanced "stickiness" of epithelial cells coated with smoke components (66); (3) enhanced pro-inflammatory responses to bacterial antigens (50); (4) reduction in anti-inflammatory IL-10 responses (37).

IL-10 appears to protect the fetus against pathogens. IL-10 knockout mice are at greater risk of some pregnancy pathologies that occur in response to infection. Low doses of endotoxin given to IL-10 knockout mice can cause fetal resorption in early pregnancy (67) and pre-term labor in late pregnancy (68). No effect on pregnancy was observed when wild-type mice were given the same dose. IL-10 acts through inhibition of inflammatory cytokines including TNFα, IFN-γ, and IL-6 (67, 69).

The *IL10*-1082A alleles have been associated with reduced production of IL-10. One SNP (G-1082A) in the promoter sequence of the *IL10* gene associated with under-expression of plasma IL-10 levels (70, 71) was present in a significantly greater proportion of ethnic groups at increased risk of stillbirths: Black Americans (45%) (36), Bangladeshis (84%), and Aboriginal Australians (83%) compared with Caucasian populations (31%) (40). Smokers had significantly lower baseline levels of IL-10 and lower responses to endotoxin than non-smokers (37). When assessed by genotype, the differences between smokers and non-smokers were significant for individuals with the heterozygous variant (GA) and the variant (AA). These data suggest interactions between cigarette smoke and genetic factors that result in reduced control of pro-inflammatory responses by IL-10.

Obesity

One of the latest meta-analyses of risk factors for stillbirths indicated that maternal overweight/obesity [body mass index (BMI) $>25\,kg/m^2$] was the highest modifiable risk factor with a population attributable risk (PAR) of 8–18% contributing to >8000 stillbirths across all high-income countries. Maternal smoking had a PAR of 4–7% contributing to more than 2800 stillbirths across all high-income countries (23). The physiological mechanisms contributing to stillbirths are not well defined; however, obesity increases the risk of gestational diabetes and hypertension. There is evidence to suggest inflammation is also involved.

Adipose tissue from lean individuals preferentially secretes anti-inflammatory adipokines such as adiponectin, transforming growth factor beta (TGFβ), IL-10, IL-4, IL-13, IL-1 receptor antagonist (IL-1Ra), and apelin. By contrast, obese adipose tissue mainly releases pro-inflammatory cytokines among which are TNF-α, IL-6, leptin, visfatin, resistin, angiotensin II, and plasminogen activator inhibitor 1 (72). In studies of obesity among Indigenous groups in which the genotype associated with higher levels of IL-6 responses is predominant, levels of this cytokine were associated with higher BMI (73, 74). Using CRP as a marker for inflammation, there is a positive correlation between BMI and CRP among adults (75). In our current studies, BMI correlated with CRP levels among Indigenous Australian women during pregnancy (Pringle, this issue).

Fetal Growth Restriction

Fetal growth restriction had the largest PAR for stillbirths in a major study of still birth risk factors in the United Kingdom (12). Down regulation of IL-10 in the placenta has been associated with IUGR in studies of a Caucasian (Swedish) (76) and an Asian (Pakistani) (77) population. Elevated CRP ($\geq 25\,mg\,L^{-1}$) was associated with lower estimated fetal weight in the third trimester and lower weight at birth and an increased risk of a small for gestational age infant (78). In a mouse model, IL-10-reduced endotoxin-induced growth retardation and fetal deaths (79); and we have found a significant correlation ($r = 0.91$) between levels of maternal and cord blood IL-10 among matched samples from elective Caesarian deliveries (49).

Both human recombinant IL-10 and the CMV IL-10 analog down regulate matrix metalloprotein 9 (MMP 9) involved in implantation. Reduced MMP 9 activity in early placenta formation has been suggested to affect cytotrophoblast remodeling of the uterine vasculature and restrict fetal growth (80). There have been no prospective studies on presence of the levels of IL-10 or the presence of CMV IL-10 analog in human pregnancy outcome. It needs to be determined if these might be associated with low-birth weight or small for gestational age infants if there is a parallel with the mouse models. The report that 15% of stillbirths in one series had evidence of CMV infection warrants further studies into the role of these infections in relation to infection and inflammation on the outcome of pregnancy, fetal survival, and health (28).

There is evidence from animal models that elevated testosterone during pregnancy results in intrauterine growth retardation (81). Among women with polycystic ovary (PCO) syndrome, maternal androgens are increased during pregnancy. At 10–16 weeks, the PCO group had higher levels of testosterone and the differences were significant at 22–28 weeks (82). In the PCO mothers, there was a higher proportion of small for gestational age infants (12.8%) compared with the control group (2.8%); and the SGA infants of the mothers with PCO were significantly smaller (83). Higher levels of testosterone during pregnancy at 17 and 33 weeks were associated with lower birth weight and length of the infant. The levels ranged from 0.5 to 7.2 $nMol\,L^{-1}$ at 13 weeks and from 0.9 to 14.5 $nMol\,L^{-1}$ at 33 weeks (84). If inflammatory responses are contributing to growth restrictions, the effects of testosterone need to be considered in the context of inflammation (62).

The Male Excess in Stillbirths

For both SIDS and stillbirths, there is a male excess. In an early analysis of the sex ratio, the authors analyzed stillbirths in the United States from 1922 to 1936. The proportion of males at <16 weeks was nearly 80% but fell to 67% at 16 weeks, a low of 53.5% at 28 weeks but rose to 57% by 36–40 weeks (16) (**Figure 2**). In a recently reported analysis of birth outcomes in Canada between 2002 and 2007, 54.8% of males were stillborn compared to 51.4% of live births (17). The greatest difference between males and females appeared to between 20 and 24 weeks gestation as noted in the earlier study.

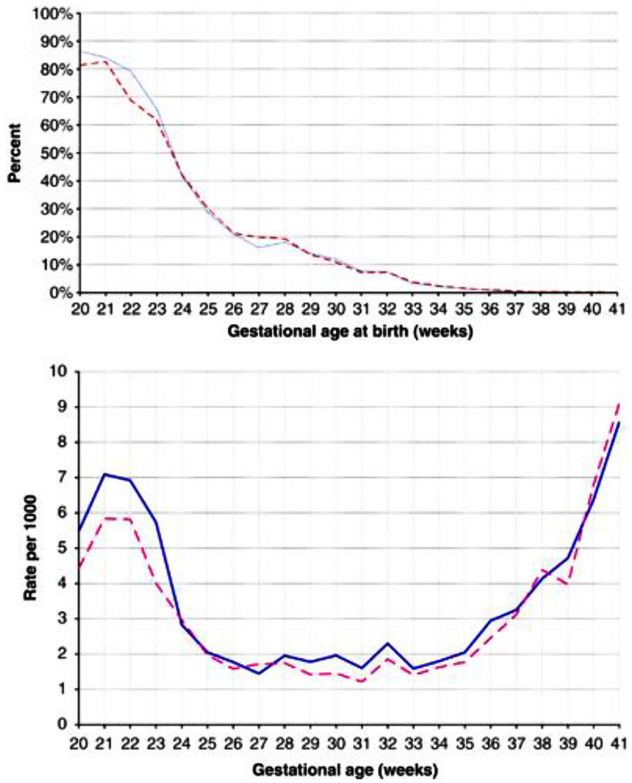

FIGURE 2 | (A) Percentage of singleton stillbirths among all singleton livebirths and stillbirths (prevalence approach). Data are for males (solid blue line) and females (dashed red line), presented by gestational age. **(B)** Number of stillbirths per 1000 singleton livebirths and stillbirths combined (incidence approach). Data are for males (solid blue lines) and female (dashed red lines), presented by gestational age. Reproduced with permission (17).

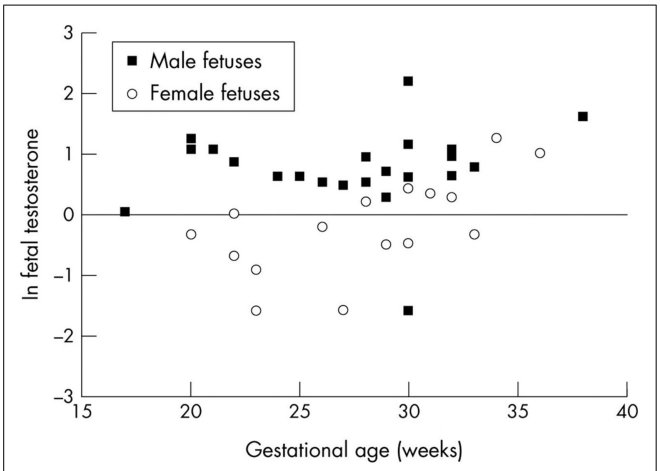

FIGURE 3 | Fetal testosterone levels, sex and gestational age.

TABLE 3 | Medians and ranges of cytokine levels (pg/ml) in amniotic fluid of male and female infants.

Cytokine	Female ($n = 12$)	Range	Male ($n = 12$)	Range
IFN-γ	1373	15–6270	2717	96–14,417
IL1-β	28	<2.4–68	41	<2.4–121
IL-6	2270	292–10,738	1218	398–12,928
IL-8	1794	578–4185	2402	621–9179
TNF-α	351	311–2025	733	24–3176
IL-10	56	9–226	112	<1.8–303
IL1-Ra*	3908	1786–6295	1839	330–5064

*$P < 0.001$.

There is a rise in testosterone production associated with the period during which SIDS is most prevalent. Between 1 and 5 months, testosterone levels range from 0.03 to 6.14 nMol L^{-1} for males and 0.03 to 0.17 for females. In males, these levels decrease to 0.07–0.24 at 6–11 months. The ranges of testosterone in the adult females (<0.4 to 3.1 nMol L^{-1}) tested in our studies were within the range for males in the 1- to 5-month age range. There was a positive correlation between testosterone levels and pro-inflammatory responses to LPS when the cells were pre-treated with IFN-γ or IFN-γ and a water soluble cigarette smoke extract (62).

Fetal plasma testosterone levels for males were significantly higher (range 1.7–2.9 nMol L^{-1}) than levels for females (range 0.45–1.3 nMol L^{-1}) (85) (**Figure 3**). If testosterone has a similar effect on inflammatory responses in the fetus, male infants might have significantly higher pro-inflammatory responses to infection or bacterial components. We found many of the pro-inflammatory cytokines are higher in the amniotic fluid of males and the anti-inflammatory IL-1Ra significantly higher in females (**Table 3**).

During the 20- to 24-week period when the difference between male and female stillbirths is most obvious, the difference in fetal testosterone levels is greatest. The testosterone levels rise significantly with gestational age among females but remain steady among males (**Figure 3**) (85). This raises the hypothesis that the higher testosterone levels present in males at 20–24 weeks gestation enhance pro-inflammatory responses as noted in our *in vitro* studies (62) and partly explain the excess of male stillbirths in this age range (**Figure 2**). Experimental systems are available to assess these interactions.

Conclusion

There is evidence from a variety of sources to suggest infection and inflammation might play a role in fetal deaths. As a variety of micro-organisms has been identified in studies of stillbirths, the common thread is most likely the effects of the inflammatory responses to infection. There is evidence to support the hypothesis that risk factors associated with stillbirths could contribute to dysregulation of the balance of inflammatory responses to infection, and these responses might trigger physiological interactions leading to fetal loss. The following recommendations are derived from the assessment of how dysregulation of the inflammatory responses could help explain the risk factors associated with stillbirths.

1. Samples from both mother and infant need to be assessed by both conventional diagnostic methods and new molecular methods for evidence of infectious agents, particularly combinations of virus and bacteria.
2. Samples from both mother and infant need to be assessed for presence of bacterial toxins, both soluble and cellular, that can act as superantigens that can induce powerful cytokine responses.

3. Direct assessment of material from both mother and infant for evidence of pro-inflammatory and anti-inflammatory cytokines is needed.
4. Determination of cotinine levels in body fluids would help determine the level of exposure to cigarette smoke.
5. For both mother and infant, determine cytokine gene polymorphisms associated with high- or low-inflammatory responses and implicated in pre-term birth.
6. Experimental studies to assess further the interactions between genetic, developmental, and environmental risk factors for their role in dysregulation of inflammatory responses that could lead to infant death.

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