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Cytokine production by bone marrow mononuclear cells in inherited bone marrow failure syndromes

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Summary

Fanconi anaemia (FA), dyskeratosis congenita (DC), Diamond-Blackfan anaemia (DBA), and Shwachman-Diamond syndrome (SDS) are characterized by the progressive development of bone marrow failure. Overproduction of tumour necrosis factor- α (TNF- α , TNF) from activated bone marrow T-cells has been proposed as a mechanism of FA-related aplasia. Whether such overproduction occurs in the other syndromes is unknown. We conducted a comparative study on bone marrow mononuclear cells to examine the cellular subset composition and cytokine production. We found lower proportions of haematopoietic stem cells in FA, DC, and SDS, and a lower proportion of monocytes in FA, DC, and DBA compared with controls. The T- and B-lymphocyte proportions were similar to controls, except for low B-cells in DC. We did not observe overproduction of TNF- α or IFN- γ by T-cells in any patients. Induction levels of TNF- α , interleukin (IL)-6 (IL6), IL-1 β , IL-10, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor in monocytes stimulated with high-dose lipopolysaccharide (LPS) were similar at 4 h but lower at 24 h when compared to controls. Unexpectedly, patient samples showed a trend toward higher cytokine level in response to low-dose (0.001 μ g/ml) LPS. Increased sensitivity to LPS may have clinical implications and could contribute to the development of pancytopenia by creating a chronic subclinical inflammatory micro-environment in the bone marrow.

Keywords

inherited bone marrow failure syndromes; immunology; cytokines; monocytes; bone marrow

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Author contributions N.G. and B.P.A. recruited patients, collected the bone marrow samples, and analysed patient data. K.M. and L.P. designed the experiments and analysed data. K.M. performed the experiments and wrote the paper. All authors provided their respective expertise and have contributed to the preparation of this paper.

Conflict-of-interest disclosure The authors declare no competing financial interests.

Supporting information Additional Supporting Information may be found in the online version of this article:

Fig S1. LPS-stimulated CD19⁺ or CD14⁺ cells do not produce IFN- γ

Fig S2. TNF- α is detected in response to low-dose LPS in patient samples.

Fig S3. IL-10, G-CSF, and GM-CSF are not induced after 4 hours of stimulation with LPS.

Introduction

Inherited bone marrow failure syndromes (IBMFS) are a group of rare genetic disorders that are associated with increased risks of developing myelodysplastic syndrome, acute myeloid leukaemia, and solid tumours (Shimamura & Alter 2010). The group includes Fanconi anaemia (FA), dyskeratosis congenita (DC), Diamond-Blackfan anaemia (DBA), and Shwachman-Diamond syndrome (SDS). Diverse sets of mutated genes with some functional overlap have been identified for these disorders (Shimamura & Alter 2010). The biological functions of these genes involve DNA repair (FA), ribosomal biogenesis (DBA, SDS, and DC [*DKC1* gene]), and telomere maintenance (DC) (Farrar & Dahl 2011; Savage & Bertuch 2010; Shimamura 2006; Shimamura & Alter 2010). Despite the differences in the affected biological functions, these syndromes are characterized by high risks of multilineage bone marrow failure (Shimamura & Alter 2010). This includes DBA, which is classically a pure red cell aplasia (Shimamura & Alter 2010), but has been reported to develop multilineage-defects in a small subset of patients (Giri *et al*, 2000). Patients with an IBMFS usually have markedly hypocellular bone marrow, even when pancytopenia is absent.

Unlike acquired aplastic anaemia, IBMFS patients usually do not respond to immunosuppressive treatment (Al-Rahawan *et al*, 2006; Bagby *et al*, 2004; Maciejewski & Risitano 2003). However, subtle immunological alterations that are often subclinical have been reported in some patients with IBMFS. Studies in FA have shown low numbers of B- and Natural Killer (NK)-cells, reduced proliferative capacities of T- and B-lymphocytes, and decreased cytotoxic activities in T- and NK-cells from peripheral blood mononuclear cells (PBMCs) (Castello *et al*, 1998; Fagerlie & Bagby 2006; Myers *et al*, 2011). Overall, the levels of immunoglobulins are generally not affected in FA patients (Myers *et al*, 2011; Roxo *et al*, 2001). An increase in tumour necrosis factor- α (TNF- α) and γ -interferon (IFN- γ) production was reported in activated bone marrow T-cells, and this overproduction was proposed as one of the key mechanisms underlying haematopoietic stem cell (HSC) aplasia (Dufour *et al*, 2003). In addition, peripheral blood monocytes from one FA patient showed a higher level of TNF- α production in response to lipopolysaccharide (LPS) (Anur *et al*, 2012). Similar to FA, defects in T- and B-cells from PBMCs have been reported in patients with SDS (Dror 2005; Dror *et al*, 2001; Khan *et al*, 2008; Shah *et al*, 2009). These defects include low IgG levels, low numbers of circulating T-, B-, and NK-cells, and proliferative defects in T- and B-cells. Similar immune defects have also been reported for DC and DBA (Cossu *et al*, 2002; Jyonouchi *et al*, 2011; Khan *et al*, 2011). These immunological alterations may have important consequences for patients with IBMFS, who may be more prone to environmental insults and are at increased risk of developing cancer. However, because the diseases are rare, the sample sizes in the aforementioned studies were relatively small, and the reported alterations were heterogeneous and inconsistent among the patients. Further studies are required to better understand the changes that occur in the immune system and the effect of these alterations in patients with IBMFS.

Prior studies of the immune system in patients with IBMFS were mostly performed on PBMCs; comprehensive studies on bone marrow cells have not been reported in the literature. Bone marrow is the major site of haematopoiesis, and B-cells and monocytes develop in this compartment. The bone marrow also contains plasma cells and memory T-cells. The bone marrow is an important tissue for immunity as it can contribute and provide support to the functions of innate and adaptive immunity (Mercier *et al*, 2012; Zhao *et al*, 2012).

Taking advantage of the IBMFS Study at the National Cancer Institute, we have conducted a comparative study of cytokine production by *in vitro*-stimulated bone marrow mononuclear cells from patients with FA, DC, DBA, or SDS. The main goal of our study was to examine

whether T-cells and monocytes from patients with IBMFS overproduce cytokines when compared with healthy individuals. Published studies have shown that TNF- α inhibits the growth of and causes apoptosis of HSCs (Broxmeyer *et al*, 1986; Dybedal *et al*, 2001). In addition, other studies have shown that T-cells and monocytes in FA patients overproduce IFN- γ and/or TNF- α (Anur *et al*, 2012; Dufour *et al*, 2003). Therefore, we hypothesized that cytokine production may be dysregulated in patients with FA and other IBMFS.

Simultaneous comparison of the immune function of bone marrow mononuclear cells from patients with FA, DC, DBA, or SDS showed that intracellular production of IFN- γ and TNF- α by activated T-cells was not higher in the syndrome groups. However, there was a trend toward enhanced innate immune responses to low-dose LPS by monocytes in patients with FA, DC, DBA or SDS.

Methods

Bone marrow aspirates and preparation of mononuclear cells

Participants were recruited under the protocols approved by the Institutional Review Board of the National Cancer Institute (NCI Protocol 02-C-0052; NCT00027274). Written informed consent was provided in accordance with the Declaration of Helsinki. Bone marrow aspirates from the posterior iliac crest were collected from March 2002 to January 2012. Bone marrow mononuclear cells from 16 FA (median age 16 years, range 3-59), 20 DC (median age 17 years, range 3-69), 21 DBA (median age 11 years, range 3-47), and 7 SDS (median age 12 years, range 6-19) patients were used in the first series of experiments (Figs 1-3). For controls, bone marrow aspirates from 14 healthy adult volunteers (median age 26 years, range 20-45) were obtained from Lonza (Walkersville, MD) in April and May of 2012. The number of cells obtained from patients was limited because of marrow hypocellularity; only 9 FA, 12 DC, 12 DBA, and 7 SDS patient samples from the initial experiments had sufficient numbers of cells for testing in the second series of experiments (Figs 4-5).

Low-density mononuclear cells were isolated using centrifugation through Ficoll-Hypaque (1.077, Sigma-Aldrich, St. Louis, MO, USA). Isolated cells were resuspended in media with L-glutamine, penicillin/streptomycin, 12.5% heat-inactivated fetal bovine serum, and 10% dimethyl sulfoxide (DMSO) (all from the American Type Culture Collection [ATCC], Manassas, VA, USA). The cells were frozen using a controlled-rate-freezer (Kryosave, Integra, Planer plc, Sunbury-on-Thames, UK), and immediately transferred into and maintained in the vapour phase of a liquid nitrogen tank.

Reagents and antibodies

Reagent resources were as follows: Phorbol 12-myristate 13-acetate (PMA), ionomycin, LPS: Sigma-Aldrich; Fluorescein isothiocyanate (FITC)-conjugated anti-CD45 and phycoerythrin (PE)-conjugated anti-CD14 Simultest: BD Biosciences (San Jose, CA, USA); Brefeldin A solution (1000 $\times$), 10 $\times$ permeabilization solution, antibodies to CD19 (PE-cyanin 7 [PC7]) and IFN- γ (Alexa Fluor 488 [AF488]): eBioscience (San Diego, CA, USA); Allophycocyanin (APC)-conjugated anti-CD34 and -CD14, PE-conjugated anti-TNF- α and -TLR4 antibodies: BioLegend (San Diego, CA, USA); and Phycoerythrin-Texas Red (ECD)-conjugated CD3: Beckman Coulter (Brea, CA, USA).

Surface and intracellular cytokine staining

Polypropylene tubes (12 $\times$ 75 mm; BD Falcon, San Jose, CA, USA) were used in the flow cytometry experiments, and samples were tested once within each experiment. Bone marrow mononuclear cells were thawed in a 37°C water bath, washed, and resuspended in AIM-V serum-free media (Invitrogen, Grand Island, NY, USA). For surface staining, the cells were

washed with wash buffer (phosphate-buffered saline with 1% heat-inactivated fetal bovine serum), and incubated with mouse IgG (Chemicon, Temecula, CA, USA) for 10 min at 4°C, then stained with anti-CD45 (FITC), -CD14 (PE), -CD3 (ECD), -CD34 (APC), and -CD19 (PC7) antibodies for 30 min at 4°C. In other experiments, the cells were stained with APC-conjugated anti-CD14 and PE-conjugated toll-like receptor (TLR) 4 antibodies for 30 min at 4°C. For the intracellular IFN- γ and TNF- α staining, the cells were incubated in AIM-V, 10 ng/ml of PMA plus 0.5 μ g/ml ionomycin, or 1 μ g/ml of LPS for 4 h at 37°C. For the dose titration experiment, intracellular cytokine staining was performed on cells stimulated with 1.0, 0.1, or 0.001 μ g/ml of LPS for 4 h. Brefeldin A was added at the start of the incubation. After washing, the cells were incubated with mouse IgG for 10 min, and stained for CD3 (ECD), CD14 (APC), and CD19 (PC7), or for CD14 (APC), at 4°C for 30 min. The samples were washed, fixed (Optilys C solution, Beckman Coulter) for 15 min on ice, washed with wash buffer and permeabilization solution, followed by a 30-min incubation with antibodies to IFN- γ (AF488) and TNF- α (PE). All samples were collected using Cytomics FC500 cytometer (Beckman Coulter), and data analysis was performed with FCS Express (De Novo Software, Los Angeles, CA, USA).

Determination of cytokine concentration

In a 96-well tissue culture plate, 0.1×10^6 mononuclear cells/well were incubated with or without three different concentrations (1.0, 0.1, or 0.001 μ g/ml) of LPS in a final volume of 200 μ l at 37°C for 4 or 24 h. Each sample was tested once. The plate was centrifuged and 160 μ l of supernatant was transferred into a new 96-well plate and stored at -80°C.

To determine the concentration of cytokines, we employed the Milliplex® Map Human Cytokine/Chemokine Magnetic Bead Panel Immunoassay Kit (Millipore, Billerica, MA, USA). We tested for IFN- γ , TNF- α , interleukin (IL)-10 (IL10), IL-1 β (IL1B), IL-6 (IL6), granulocyte colony-stimulating factor (G-CSF, CSF3) and granulocyte-macrophage colony-stimulating factor (GM-CSF, CSF2), which are known to be secreted by LPS-stimulated monocytes (Rossol *et al*, 2011). In brief, the samples and standards were tested in duplicates of 25 μ l in an assay-specific 96-well plate. Assay buffer (25 μ l) was added to the sample wells and 25 μ l AIM-V to the standard, followed by 25 μ l of a cocktail of antibody beads that recognize the cytokines. The plate was protected from light and incubated at 4°C overnight on a shaker. The next day, after washing, 25 μ l of detection antibody was added, and the samples were incubated at room temperature for 1 h on a shaker. Without washing, an additional 25 μ l of streptavidin-phycoerythrin was added, incubated on a shaker for 30 min, washed, and resuspended in 150 μ l of buffer before the concentrations were measured using the Bioplex 200 System (Bioplex Manager 6.1, Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed using JMP (SAS, Cary, NC, USA). The statistical significance between IBMFS and the controls was determined using the two-tailed, Mann-Whitney non-parametric test with an alpha level of 0.05.

Results

Proportions of bone marrow cells

B cells and monocytes develop in the bone marrow from CD34⁺ HSCs (Mercier *et al*, 2012; Zhao *et al*, 2012). The bone marrow also contains memory T-cells (Di Rosa & Pabst 2005; Masopust & Picker 2012; Wood *et al*, 2007). We assessed the proportions of HSC, monocyte, T-cell, and B-cell populations in bone marrow mononuclear cells from 16 FA, 20 DC, 21 DBA, and 7 SDS patients, and 14 healthy adult controls by flow cytometry. The percentages of HSCs were significantly lower than controls in FA, DC, and SDS, but not in

DBA (Fig 1A). The percentages of monocytes were below those of controls in FA, DC and DBA, but not in SDS (Fig 1B). The only difference in the lymphocyte populations was a lower proportion of B-cells in DC than in controls (Fig 1C-D). The low proportions of HSCs in FA, DC, and SDS were consistent with published studies (Ceccaldi *et al*, 2012; Dror 2005; Dror & Freedman 1999; Dror *et al*, 2001; Goldman *et al*, 2008). The low percentage of monocytes in DBA suggests that a lineage defect downstream of HSCs may occur in these patients (Giri *et al*, 2000). Thus, the proportions of HSCs and monocytes were generally low in IBMFS, while T- and B-cells were generally comparable to those in controls.

Intracellular TNF- α and IFN- γ production in T cells

Increased production of TNF- α and IFN- γ by stimulated T-cells has been reported following pharmacological stimulation of bone marrow mononuclear cells from patients with FA; this overproduction was proposed as a key mechanism of bone marrow aplasia (Dufour *et al*, 2003). To test whether T-cells in our patients with FA and in those with other IBMFS overproduce these cytokines, we stimulated the bone marrow mononuclear cells with PMA plus ionomycin (P+I) and measured intracellular TNF- α and IFN- γ by flow cytometry. Both cytokines were produced in all groups, including the control, after 4 h of stimulation (Fig 2A). FA, DC, and SDS were comparable to the control, while DBA had lower proportions of IFN- γ -positive and TNF- α -positive T-cells (Fig 2B-C).

Detection of intracellular TNF- α in monocytes stimulated with LPS

Peripheral blood monocytes from one FA patient have been reported to overproduce TNF- α (Anur *et al*, 2012). Therefore, we tested the ability of CD14⁺ bone marrow monocytes to produce TNF- α and IFN- γ in response to LPS. In addition, we also examined the production of these cytokines by B-cells, because they could potentially respond to LPS. Mature human B-cells from healthy individuals generally do not respond well to LPS; however, TLR 4, a receptor responsible for responding to LPS (Rossol *et al*, 2011), is upregulated in patients with inflammatory diseases (Ganley-Leal *et al*, 2010). Because immunological alterations have been reported in patients with IBMFS, we wanted to test whether CD19⁺ cells in the bone marrow could respond to this stimulus. TNF- α (Fig 3A) or IFN- γ (Fig S1) was not detected after 4 h of stimulation with 1 μ g/ml LPS in B-cells. The cells responsible for producing TNF- α were monocytes (Fig 3B) and, as expected, they did not produce IFN- γ (Fig S1). Only a minimal amount of TNF- α was detected in the no-stimulation samples from any group (Fig 3B). We found that, within the monocyte population, the percentages of TNF- α -producing CD14⁺ cells were lower in FA and DBA than in the controls (Fig 3C). Our data showed that monocytes were the major producers of TNF- α in response to LPS. Together with the data in Fig 2, we found that T-cells or monocytes from patients with IBMFS do not overproduce IFN- γ or TNF- α .

Sensitivity of monocytes to low-dose LPS

Because LPS is a more specific stimulus than P+I, and because it signals through TLR4 (Rossol *et al*, 2011), we surmized that the LPS-related differences in cellular response were likely to be more physiologically relevant. To further ascertain whether the response of monocytes in FA or DBA patients was weaker than the controls, we conducted dose-response experiments to determine whether cytokines secreted in response to varying doses of LPS differed between the IBMFS and the control groups. We also examined the production of intracellular TNF- α and IFN- γ . All samples were tested for the measurement of cytokines in response to all doses of LPS (1.0, 0.1, or 0.001 μ g/ml) at 4- and 24-h time points. Because the number of cells available for analysis was limited, not all patient

samples were tested in the intracellular staining experiment. In some samples, we were able to test only the highest and the lowest doses.

We detected TNF- α -producing monocytes in response to 1 μ g/ml of LPS in all groups, and the proportions were comparable between each syndrome and the control group (Fig 4A-B). In this experiment, there were no significant differences between FA or DBA and controls, while TNF- α -producing monocytes were significantly lower in Fig 3C. The different results between these two experiments are probably due to the smaller sample size in Fig 4B. Stimulation with 0.1 μ g/ml of LPS showed cellular responses similar to those stimulated with 1 μ g/ml of LPS (Fig 4A-B). However, intracellular TNF- α was detected in response to 0.001 μ g/ml of LPS in some patient samples (Fig 4A) with levels that were significantly greater in FA, DBA, and SDS than in the controls (Fig 4B).

We hypothesized that the expression level of TLR4 could be responsible for the differential response observed in these two experiments. However, when we examined the median fluorescent intensity (MFI) of TLR4 on unstimulated monocytes, we did not find any difference between the groups (Fig 4C), suggesting that the expression level of TLR4 was not responsible for the increased sensitivity to LPS in monocytes from patients with IBMFS.

Secretion of TNF- α in response to varying doses of LPS

To determine the amount of cytokines secreted in response to LPS, we employed the multiplex Luminex technology to measure the concentrations of IFN- γ and TNF- α in the cell culture supernatants at 4- and 24-h time points. IFN- γ was not detected, even after 24 h of stimulation (data not shown). In contrast, we detected substantial amounts of TNF- α in all groups after 4 h of stimulation with high doses (1 and 0.1 μ g/ml) of LPS (Fig S2). The concentrations of TNF- α detected for these 2 doses were generally similar within each group and between the syndrome groups. However, the concentrations were lower in IBMFS patient samples than in the controls (Fig S2), a result possibly attributable to the lower number of monocytes in patients (Fig 1B). Despite the low numbers, higher concentrations of TNF- α were detected in some patient samples when the cells were stimulated with low-dose (0.001 μ g/mL) LPS. In contrast, only minimal amounts of TNF- α were detected in the controls (Fig S2). A similar pattern of results was observed at the 24-h time point, except that the concentrations were higher than those from the 4- time point (Fig S2).

Determination of TNF- α induction in response to LPS

To minimize the difference in the number of monocytes, we calculated stimulation indexes (fold-increase over no-stimulation controls) to evaluate the induced level of TNF. At the 4-h time point, TNF- α was greatly increased in response to the high doses of LPS in all the groups, and the levels of induction were comparable between the groups (Fig 5A). Also, the levels of induction were similar for these 2 doses of LPS; however, after low-dose stimulation, the control group showed minimal induction of TNF- α *versus* higher induced levels in FA, DC, and SDS (Fig 5A). Although the induction level was further increased in all groups at all doses at 24 h, it was lower in IBMFS than in controls at the high doses. At the low-dose, we observed a trend toward higher TNF- α induction in FA, DC, and SDS (Fig 5A).

Induction levels of IL-6, IL-1 β , IL-10, G-CSF, and GM-CSF

In addition to IFN- γ and TNF- α , we also measured the concentrations of IL-6, IL-1 β , IL-10, G-CSF, and GM-CSF, and examined the induction levels of these cytokines using the stimulation index. While moderate-to-strong inductions were observed for IL-6 and IL-1 β , IL-10, G-CSF, and GM-CSF were minimally detected at 4 h (Fig S3).

We found patterns and trends of induction that were similar to those for TNF- α . At the high doses, the induction levels of IL-6 and IL-1 β were comparable among different groups at 4 h, except for SDS, which showed higher responses than the control (Fig 5B). The levels of the majority of the cytokines in IBMFS were generally below those of the controls at 24 h (Fig 5C). As in our data with TNF- α , stimulation with the low-dose LPS led to the secretion of cytokines from patient samples, and there was a general trend toward higher induction in FA, DC, and SDS (Fig 5B-C). This was particularly prominent for SDS and FA at both time points. In contrast, DBA and the control showed minimal inductions at both time points (Fig 5C). Collectively, our data showed that the cells from IBMFS bone marrows produced the expected cytokines in response to LPS. More important, the data in Figs 4 and 5 showed that monocytes in IBMFS are more sensitive to low-dose LPS, and an overall trend toward greater cytokine induction was observed.

Discussion

We present here the first study of cytokine production in the bone marrow of patients with one of the four major inherited bone marrow failure disorders (FA, DC, DBA, SDS). There are two major findings: (1) T-cells did not overproduce either TNF- α or IFN- γ , and (2) monocytes were more sensitive to low-dose LPS stimulation in patients with IBMFS. Contrary to our expectation, bone marrow lymphocyte percentages were comparable to those in controls. Nevertheless, the absolute cell numbers could still be lower in patients with IBMFS because of the bone marrow hypocellularity. Additionally, because the T-cell precursors originate from HSCs in the bone marrow, development of new naive T-cells in the thymus may be affected in patients with IBMFS. Because we used only CD19 as our B-cell marker, we could not differentiate recirculating mature B-lymphocytes from those that were going through B-cell development. To address these questions, absolute cell numbers and the percentages of different cell populations should be measured in future samples at the time of collection. Flow cytometry can be used to distinguish subpopulations of B-cells (Jackson *et al*, 2008). Furthermore, T-cell receptor excision circle analysis should provide useful information on the presence of newly selected T-cells (Somech 2011).

Based on the previous study that showed T-cells overproduce TNF- α (Dufour *et al*, 2003), etanercept, a TNF- α inhibitor, was administered to patients with FA in a clinical trial to ameliorate bone marrow failure (Mehta *et al*, 2012), a study which failed to demonstrate patient benefit. Our data, which showed no evidence of TNF- α overproduction by activated T cells, were consistent with that null result. Moreover, we observed that the levels of serum TNF- α in patients with IBMFS, including FA, were comparable to those in controls (unpublished observations). However, we cannot rule out the existence of functional alterations in T-cells in IBMFS. From the current study, it is difficult to assess whether cytokine production is affected, because we employed a non-physiological stimulus. We used P+I, a pharmacological stimulus, because the previous study also used a similar pharmacological stimulus to stimulate T-cells. Perhaps, the use of anti-CD3 antibody or antigens might better reveal functional alterations in T-cells. In DBA, haploinsufficiency in ribosomal function may be related to suboptimal cytokine production that cannot be overcome even by the potent P+I stimulus.

We have concentrated our efforts on studying the innate immune responses that are mediated through TLR4. Dose titration of LPS revealed the hypersensitive nature of bone marrow CD14⁺ monocytes in IBMFS. A similar finding has been reported (Anur *et al*, 2012), in which monocytes purified from PBMCs of one FA patient produced more TNF- α than the control cells in response to 0.1 ng/ml of LPS. In that study, however, dose titration was not reported. Our data provide further support of that observation, and extend the observation of hypersensitivity to other IBMFS.

The high doses of LPS provided a maximal level of activation, and the cells probably reached their saturation point in responding to the stimulus. However, under limiting conditions, cells from IBMFS patients were more sensitive to LPS than those from controls. It was intriguing that the amount of TNF- α secreted by cells of DBA patients was minimal in response to the low-dose LPS, yet we were able to detect an intracellular level of this cytokine. In monocytes, the release of membrane-bound TNF- α is mediated by tumour necrosis factor- α -converting-enzyme (TACE) (Black *et al*, 1997). It may be that TACE is under-expressed in DBA cells or that these cells have inefficient protein trafficking and/or secretory pathway. To investigate these potential mechanistic questions, future studies using quantitative polymerase chain reaction, biochemical analysis, and microscopy are needed. The cells of FA, DC, and SDS patients showed a similar tendency toward higher cytokine induction at the low-dose LPS. Considering the various biological functions of the genes that are mutated in these syndromes, there are no obvious explanations for this hypersensitivity. Other than SDS and DC with mutation in the *DKC1* gene, the functions of these gene products are not directly involved in protein synthesis. For SDS, this finding is counterintuitive, because the maturation of the 60s ribosomal protein is compromised in this syndrome (Dokal & Vulliamy 2010; Parikh & Bessler 2012). It is also unclear why these different syndromes show similar functional alterations. One possible explanation is that cells of these syndromes may have alterations in p53 (TP53). These IBMFS have been reported to show dysregulation of p53 (Bellodi *et al*, 2010; Ceccaldi *et al*, 2012; Chin *et al*, 1999; Elghetany & Alter 2002; Narla & Ebert 2010; Zhang & Lu 2009). Genome instability that leads to a quantitatively and/or qualitatively elevated level of p53 might initiate a compensatory/feedback mechanism in cells to upregulate a survival factor, such as NF- κ B (NFKB1). As NF- κ B is an important transcription factor for the expression of proinflammatory cytokines, its enhanced level and/or activity could potentially explain why different syndromes with different gene mutations and different molecular pathways exhibit a similar functional phenotype. It would be worthwhile to examine whether treatment of cells with a p53 inhibitor, such as pifithrin, or with an RNA interference agent could reduce the production of cytokines.

Furthermore, whether the mutated genes and altered pathways are directly responsible for this hypersensitivity is unclear. At least for TNF- α in FA, constitutive overproduction of this cytokine by EBV-transformed human lymphoblast mutant cell lines of *FANCA* and *FANCC* genes was corrected after the cells were reconstituted with the wild-type genes (Anur *et al*, 2012; Briot *et al*, 2008). Overproduction of TNF- α has also been observed in other cell lines that contain mutations in *FANCB*, *BRAC2* (previously termed *FANCD1*), *FANCF* and *FANCG* genes (Briot *et al*, 2008; Rosselli *et al*, 1994). In some of the mutant cell lines, NF- κ B and mitogen-activated protein kinase pathways were aberrantly activated (Anur *et al*, 2012; Briot *et al*, 2008). These pathways may be dysregulated in FA, SDS, and DC. Similar experiments to those performed on FA, such as establishment of mutant cell lines and reconstitution with the respective wild type genes, combined with biochemical analysis, could certainly provide insights into molecular defects that are associated with the other syndromes. These experiments in combination with microarray technology could possibly identify dysregulated genes and altered pathways. Another possibility is that monocytes in some patients may have been presensitized by IFN- γ . Such sensitized cells have been reported to show enhanced production of TNF- α following LPS stimulation (Hayes *et al*, 1995). This may explain why the hypersensitivity in the syndrome groups is heterogeneous. Perhaps microarray analysis could potentially address this question. Alternatively, heterogeneous response may be the result of polymorphism in the *TLR4* gene. In IBMFS patients, *TLR4* may have mutations that lead to enhanced response. Single nucleotide polymorphism analysis can help shed light on the status of *TLR4*, as well as other *TLR* and *TP53* genes, in these patients.

The majority of the patient samples we tested were from children (< 18 years of age), while the control bone marrow samples were obtained from adult volunteers. The literature indicates that the immune responses in healthy children, including those of the innate immune cells, are not elevated when compared with adults (Baehner & Johnston 1972; Buck *et al*, 2002; Chipeta *et al*, 1998; Gardner *et al*, 1981; Hartel *et al*, 2005; Kollmann *et al*, 2012). Thus, this age difference is probably not the major reason for the hypersensitivity. As in the reported studies on IBMFS, the sample size in each syndrome group was relatively small, and the responses were heterogeneous in our study. Additionally, because this was a cross-sectional study, longitudinal studies may help us understand the changes that occur in cells over time and clarify their potential role in the development of bone marrow failure.

Overall, our data are consistent with the notion that the immunological alterations in patients with IBMFS are subtle and largely subclinical. A low-level, chronic adverse environmental exposure could potentially trigger a response from monocytes in patients with IBMFS and incrementally contribute to the bone marrow failure. While a single event may not be sufficient to acutely modulate haematopoiesis, repeated exposure, combined with innate hypersensitivity, may lead to high local cytokine concentrations and to the development of aplasia by affecting HSC division and differentiation.

Our data do not permit defining specific therapeutic implications at present. While our findings are intriguing, additional research using complementary approaches is required to more precisely define the underlying molecular mechanisms of this intrinsic hypersensitivity, and to delineate its implications for the treatment of bone marrow failure. We believe that focusing on identifying therapeutic targets within TLRs and the signalling pathways that are involved in secretion of proinflammatory cytokines might be more beneficial than directly targeting cytokines. Because of the heterogeneity of responses by different patients, multiple different proteins may need to be targeted for an immunologically targeted therapy to be effective. As more data become available on whether reconstitution of mutated genes in IBMFS will ameliorate the overproduction of cytokines, gene therapy could be considered as an option for patients with IBMFS.

In this study, we only examined the cellular response to LPS, which signals through TLR4; however, these cells can respond to pathogens, such as viruses, through other TLRs. Dysregulation of TLR8-mediated production of TNF- α has been reported in *FANCA*- and *FANCC*-mutant cells (Anur *et al*, 2012), suggesting that innate immune responses through other TLRs may also be dysregulated in monocytes in patients with IBMFS. Further studies that use ligands specific for each TLR are required to characterize whether innate immune responses through other TLRs are altered in patients with IBMFS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Patients with IBMFS have lower percentages of HSCs and monocytes than the controls. Bone marrow mononuclear cells from patients with FA, DC, DBA, or SDS, and healthy adult controls were analysed by flow cytometry. The cells were surface stained for the pan-leucocyte marker CD45 and for markers of leucocyte subsets. The plots show percentages of double-positive cells in the total bone marrow mononuclear cells found in a live cell-gate, which was based on the forward and side scatter characteristics. (A) The percentages of CD34⁺ (HSCs) cells were lower than the controls in FA, DC, and SDS. (B) The percentages of CD14⁺ (monocytes) cells were lower in FA, DC, and DBA than in the controls. (C) The percentages of CD3⁺ (T-lymphocytes) cells were similar to the controls in all IBMFS. (D) The percentage of CD19⁺ (B-lymphocytes) cells was lower in DC than in the controls. Each symbol represents an individual, and the horizontal bars show the means. Asterisks indicate statistical significance ($p < 0.05$).

IBMFS, inherited bone marrow failure syndrome; FA, Fanconi anaemia; DC, dyskeratosis congenita; DBA, Diamond-Blackfan anaemia, SDS, Shwachman-Diamond syndrome; Ctrl, control; HSCs, haematopoietic stem cells.

Fig 2.

The percentages of IFN- γ ⁺ or TNF- α ⁺ CD3⁺ T cells in patients with IBMFS are not higher than the controls. Bone marrow mononuclear cells were incubated with or without P+I for 4 h, surface stained for CD3, and then for intracellular IFN- γ and TNF- α .

(A) Representative dot plots from single individuals in each group showed that stimulated T cells produced IFN- γ and TNF- α in all groups. (B-C) The production of IFN- γ and TNF- α was not increased in any of the syndromes; it was decreased in DBA.

IBMFS, inherited bone marrow failure syndrome; FA, Fanconi anaemia; DC, dyskeratosis congenita; DBA, Diamond-Blackfan anaemia, SDS, Shwachman-Diamond syndrome; Ctrl, control; IFN- γ , γ -interferon; TNF- α , tumour necrosis factor- α ; P+I, Phorbol 12-myristate 13-acetate + ionomycin

Fig 3.

The proportions of TNF- α -producing CD14⁺ monocytes are lower than in the controls in FA and DBA. Bone marrow mononuclear cells were stimulated with 1 μ g/ml of LPS for 4 h and stained for CD19 and CD14, and then for intracellular TNF- α . (A) Representative dot plots from single individuals showed LPS-stimulated CD19⁺ B cells do not produce TNF- α . (B) CD14⁺ monocytes in the same samples shown in (A) produced TNF- α in all groups. (C) Lower proportions of monocytes in FA and DBA produced TNF- α .

IBMFS, inherited bone marrow failure syndrome; FA, Fanconi anaemia; DC, dyskeratosis congenita; DBA, Diamond-Blackfan anaemia, SDS, Shwachman-Diamond syndrome; Ctrl, control; IFN- γ , γ -interferon; TNF- α , tumour necrosis factor- α ; LPS, lipopolysaccharide.

Fig 4.

CD14⁺ monocytes from the majority of IBMFS patients are hypersensitive to low-dose LPS. Bone marrow mononuclear cells were stimulated with 1.0, 0.1, or 0.001 µg/mL of LPS for 4 h and stained for CD14, then for intracellular TNF-α. All 14 samples in the control group were tested for each stimulation condition. For the syndrome groups, the following numbers of samples were tested: no-stimulation, 0.001 and 1.0 µg/ml LPS: FA (*N* = 9/9); DC (*N* = 7/12); DBA (*N* = 12/12); and SDS (*N* = 7/7); 0.1 µg/ml LPS: FA (*N* = 7/9); DC (*N* = 7/12); DBA (*N* = 11/12); and SDS (*N* = 4/7). (A) Dot plots of single individuals from each group are shown. All samples responded to 1 and 0.1 µg/ml of LPS, but only IBMFS patients responded to 0.001 µg/ml. Individuals who responded to the lowest dose of LPS are presented in this panel. (B) Higher proportions of CD14⁺ monocytes produced TNF-α in FA, DBA, and SDS in response to 0.001 µg/mL of LPS. (C) The median fluorescent intensity of TLR4 on CD14⁺ cells was comparable among the groups.

IBMFS, inherited bone marrow failure syndrome; FA, Fanconi anaemia; DC, dyskeratosis congenita; DBA, Diamond-Blackfan anaemia, SDS, Shwachman-Diamond syndrome; Ctrl, control; IFN-γ, γ-interferon; TNF-α, tumour necrosis factor-α; LPS, lipopolysaccharide.

Fig 5.

Cells from patients show a trend toward cytokine induction in response to low-dose LPS. Determination of cytokine concentrations in the LPS-stimulated culture supernatants was performed as described in Methods. To normalize for the difference in the number of CD14⁺ monocytes, we calculated stimulation indexes to determine the fold-increase over the no-stimulation controls. The levels of cytokines other than TNF- α in the no-stimulation controls were below the limit of detection. For these cytokines, an average number (12 assays) of the lowest concentration detected for each standard was used as the value for the no-stimulation control to calculate the stimulation index: IL-6 (3.1 pg/ml); IL-1 β (3.18 pg/ml); IL-10 (2.89 pg/ml); G-CSF (3.00 pg/ml); and GM-CSF (3.19 pg/ml). (A) The mean fold-increase in TNF- α after 4 and 24 h of stimulation showed a trend toward higher induction in FA, DC, and SDS at the lowest dose of LPS. (B) Inductions of IL-6 and IL-1 β were similar between the groups, but SDS was higher at 4 h. (C) The induction levels of IL-6, IL-1 β , IL-10, G-CSF, and GM-CSF after 24 h of LPS stimulation were generally lower in IBMFS than in the controls at the high doses, but greater at the lowest dose in FA, DC, and SDS. IBMFS, inherited bone marrow failure syndrome; FA, Fanconi anaemia; DC, dyskeratosis congenita; DBA, Diamond-Blackfan anaemia, SDS, Shwachman-Diamond syndrome; Ctrl, control; IFN- γ , γ -interferon; TNF- α , tumour necrosis factor- α ; LPS, lipopolysaccharide; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.