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Mannose-binding lectin deficiency influences innate and antigen-presenting functions of blood myeloid dendritic cells

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Summary

Mannose-binding lectin (MBL) is a serum lectin that plays a significant role in innate host defence. Individuals with mutations in exon 1 of the *MBL2* gene have reduced MBL ligand binding and complement activation function and increased incidence of infection. We proposed that, during infection, MBL deficiency may impact on dendritic cell (DC) function. We analysed the blood myeloid DC (MDC) surface phenotype, inflammatory cytokine production and antigen-presenting capacity in MBL-deficient (MBL-D) individuals and MBL-sufficient (MBL-S) individuals using whole blood culture supplemented with zymosan (Zy) or MBL-opsonized zymosan (MBL-Zy) as a model of infection. Zy-stimulated MDCs from MBL-D individuals had significantly increased production of interleukin (IL)-6 and tumour necrosis factor (TNF)-α. Stimulation with MBL-Zy significantly decreased IL-6 production by MDCs from MBL-D, but had no effect on TNF-α production. MDCs from both MBL-S and MBL-D individuals up-regulated expression of the activation molecule CD83, and down-regulated expression of homing (CXCR4), adhesion (CD62L, CD49d) and costimulatory (CD40, CD86) molecules in response to Zy and MBL-Zy. MDC from both MBL-D and MBL-S individuals induced proliferation of allogeneic (allo) T cells following Zy or MBL-Zy stimulation; however, MBL-D individuals demonstrated a reduced capacity to induce effector allo-T cells. These data indicate that MBL deficiency is associated with unique functional characteristics of pathogen-stimulated blood MDCs manifested by increased production of IL-6, combined with a poor capacity to induce effector allo-T-cell responses. In MBL-D individuals, these functional features of blood MDCs may influence their ability to mount an immune response.

Keywords: cytokines; dendritic cells; innate immunity; interleukin-6; mannose-binding lectin

Introduction

Mannose-binding lectin (MBL) belongs to a family of soluble pattern recognition molecules called collectins that elicit diverse biological activities. Via its multiple carbohydrate-recognition domains, MBL binds to mannose and N-acetyl-glucosamine oligosaccharides present on the surface of bacteria, fungi and yeast.[1–5] Following pathogen recognition, MBL activates the complement system in an antibody- and C1-independent process involving MBL-associated serine proteases, thereby facilitating pathogen removal.[6] MBL also facilitates phagocytosis of cellular debris and may therefore prevent autoimmunity.[7,8]

A deficiency in the function and level of MBL (defined as < 0·5 μg/ml MBL mannan binding and < 0·2 U C4/μl) is found in 25% of otherwise healthy individuals,[9,10]

Abbreviations: MBL, mannose-binding lectin; MBL-D, mannose-binding lectin-deficient (< 0·5 μg/ml MBL and < 0·2 U C4/μl); MBL-S, mannose-binding lectin-sufficient (> 0·5 μg/ml MBL and > 0·2 U C4/μl); Zy, zymosan ; MBL-Zy, MBL-opsonized zymosan.

representing the most prevalent innate immune deficiency. MBL deficiency results from the combined effect of multiple structural and promoter polymorphisms in the human *MBL2* gene. Three structural mutations in exon 1 have been reported (codon 54, Gly–Asp; codon 57, Gly–Glu; codon 52, Arg–Cys),[11–13] commonly referred to as variants *B*, *C* and *D*, respectively, with variant *A* being the wild type. Several polymorphisms in both the promoter and 5′ untranslated regions of the *MBL2* gene are in absolute linkage disequilibrium with coding variants leading to seven commonly expressed haplotypes: *HYPA*, *LXPA*, *LYQA*, *LYPA*, *HYPD*, *LYPB* and *LYQC*.[14,15]

MBL deficiency is a risk factor for the development of infection. Human cohort studies have described associations between MBL deficiency and susceptibility to meningococcal meningitis,[16,17] human immunodeficiency virus (HIV) infection,[18] hepatitis C virus (HCV) infection[19,20] and severe bacterial and fungal infections producing sepsis.[21–23] MBL deficiency has also been associated with increased frequencies of bacterial, viral and fungal infections in both children and adults,[24–28] particularly following bone marrow transplantation.[29–31]

The role of MBL as a modulator of infection appears to be complex and, accordingly, its mechanism of action remains incompletely characterized. Infection studies utilizing MBL null murine models contribute to knowledge of the function of MBL in the immune system. Shi *et al.*[32] demonstrated a key role for MBL in *Staphylococcus aureus* pathogenesis, particularly in combination with chemotherapy-induced neutropenia. A more recent study demonstrated a role for MBL in susceptibility to post-burn infection with *Pseudomonas aeruginosa*.[33] In both cases, the susceptibility conferred by the null genotype was reversed by administration of recombinant MBL.[32,33] Studies of human immune cells suggest that MBL deficiency may influence proinflammatory cytokine production in monocytes, peripheral blood mononuclear cells and neutrophils[34–37] as well as neutrophil superoxide production.[38]

Dendritic cells (DCs) are antigen-presenting cells which are central to the induction of antigen-specific immune responses.[39,40] To induce antigen-specific immune responses, DCs are required to recognize and process foreign material and present antigenic peptides, costimulatory molecules and cytokines to cells of the adaptive immune system: namely to T and B lymphocytes.[41] Recognition of foreign antigens and subsequent delivery of costimulatory and cytokine signals are regulated by interactions between pathogen and pathogen recognition receptors (PRRs) which are expressed by DCs, or present as soluble plasma pattern recognition proteins, including MBL.

To date, the effects of MBL deficiency on blood DC activation and subsequent T-cell responses that would determine an individual's ability to respond appropriately to an infectious episode have not been investigated. Given that DCs are antigen-presenting cells with potent capacity to respond to microbial stimulation, and are central to the induction of antigen-specific immune responses,[39,40] we hypothesized that MBL deficiency may impact on DC function induced by pathogen stimulation. To test this hypothesis, we compared the surface phenotype, inflammatory cytokine production and antigen-presenting capacity of blood myeloid (M)DCs of MBL-deficient (MBL-D) individuals with those of MBL-sufficient (MBL-S) individuals following stimulation with zymosan (Zy). Our data indicate that blood MDCs from MBL-D individuals, unlike those from MBL-S individuals, displayed unique functional characteristics, including higher production of proinflammatory cytokines interleukin (IL)-6 and tumour necrosis factor (TNF)-α, but poor capacity for allogeneic (allo)-T-cell effector cell induction. These differences in both innate and antigen-presenting functions of MDCs may contribute to increased infection and poor clinical outcome in MBL-D individuals.

Materials and methods

Study population and criteria for selection of MBL-D and MBL-S individuals

Healthy adult donors were recruited from the Mater Medical Research Institute (MMRI) volunteer blood donor database with informed consent according to the Mater Adult Hospital Ethics Committee Guidelines (MMRI#34). The mannan-binding MBL level and C4 deposition were analysed by enzyme-linked immunosorbent assay (ELISA) using established protocols.[10] The *MBL2* genotype was determined using single specific primer polymerase chain reaction (SSP-PCR) according to the established protocol.[10,42] MBL-D individuals were defined as having functional MBL level $< 0{\cdot}5$ μg/ml and C4 deposition $< 0{\cdot}2$ U C4/μl. MBL-S individuals were defined as having MBL function level $> 0{\cdot}5$ μg/ml and C4 deposition $> 0{\cdot}2$ U C4/μl. Other donor characteristics such as age, sex and ethnicity were de-identified from the researchers, and individuals were selected for further study based on volunteer availability for blood donation.

Monoclonal antibodies

The following monoclonal antibodies (mAbs) were used in experiments where specified. Mouse anti-human monoclonal antibodies were as follows: fluorescein isothiocyanate (FITC)-conjugated mAbs specific for CD86, CD15 and immunoglobulin G1 (IgG1) isotype control; phycoerythrin (PE)-conjugated mAbs specific for CD14, CD56, CD19, CD34, CD40, CD49, CD83, CD86, CD123, CD62L, chemokine (C-X-C motif) receptor 4 (CXCR4) and IgG1 isotype control; peridinin chlorophyll protein (PerCP)-conjugated mAbs specific for human leucocyte antigen (HLA)-DR and IgG1 isotype control; allophycocyanin (APC) mAbs specific

for CD14, CD11c and IgG1 isotype control (all from BD Biosciences, Sydney, Australia). Rat anti-human PE-conjugated mAbs specific for IL-2, IL-10, IL-6 and IgG isotype control and mouse-anti-human PE-conjugated mAbs specific for IL-4, interferon (IFN)-γ, IL-12 p40/p70, TNF-α and IgG isotope controls were also obtained from BD Biosciences. Mouse anti-human FITC- or PE-conjugated mAbs specific for CD83 were obtained from Immunotech (Sydney, Australia). Sheep anti-mouse (SAM) FITC-conjugated and PE-conjugated IgF(ab)$_2$ fragment was obtained from Silenus (Melbourne, Australia). Mouse anti-human unconjugated mAbs specific for CD14, CD19, CD20 and CD56 were obtained from Beckman Coulter (Gladesville, NSW, Australia); those specific for CD34, CD325a and CD66 were from BD Biosciences. Mouse anti-human unconjugated mAbs specific for CD3 (clone OKT3), CD8 (clone OKT8) and CD45RO (clone UCHL-1) from MMRI Brisbane, Australia. Mouse anti-human FITC-conjugated mAb specific for MBL (clone 131-01) was obtained from BioPorto (Gentofte, Denmark).

Preparation of Zy and MBL opsonization of Zy

Zy (from *Saccharomyces cerevisiae*; Molecular Probes, Mount Waverly, Australia) was re-suspended at 20 mg/ml in phosphate-buffered saline (PBS) containing 0·01% azide according to the manufacturer's instructions. One millilitre of purified human plasma MBL (hpMBL) (Cooperative Research Centre-Vaccine Technology, Australian Red Cross Blood Service, 44 Musk Avenue, Kelvin Grove, QLD, Australia) at 5 μg/ml in Tris-BSA [20 mM Tris–Cl, pH 8·0, 150 mM NaCl and 0·5% bovine serum albumin (BSA)] (JRH Biosciences, Brooklyn, Australia), 5 mM $CaCl_2$ (Ajax Finechem, Taren Point, Australia) and 2 mM $MgCl_2$ (BDH/VWR International, Arlington Heights, IL, USA) was mixed with 100 μl of Zy and incubated for 15 min at 37°, 5% CO_2. Zy was pelleted by centrifugation at 1000 ***g*** for 5 min and washed twice in Tris-BSA. Zy without addition of MBL was prepared in parallel using the same procedure. MBL opsonization of Zy (hereafter referred to as MBL-Zy) was confirmed by staining with FITC-conjugated mAb specific for MBL and analysed by flow cytometry (Fig. 1).

Cell culture

Whole blood was collected in ethylenediaminetetraacetic acid (EDTA) or citrate phosphate dextrose-adenine (CPDA), diluted 1 : 1 [volume/volume (v/v)] in RPMI-1640 culture medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Gibco, Invitrogen, Mount Waverly, Australia) and cultured in a tissue culture flask (200 ml of diluted blood) or a 24-well plate (2 ml of diluted blood/well) without microbial stimuli, with Zy (100 μg/ml blood), MBL-Zy (100 μg/ml blood) or lipopolysaccharide (LPS; 1 μg/ml blood; Sigma, St Louis, MO) for 6 hr. Blood was harvested from tissue culture flasks and peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll gradient (Amersham Biosciences, Rydalmere, Australia). GolgiPlug (1 μl/ml culture; BD Biosciences) was added to blood cultured in 24-well plates for the last 5 hr of culture, and blood was harvested and assayed for cytokine production by blood MDCs and monocytes.

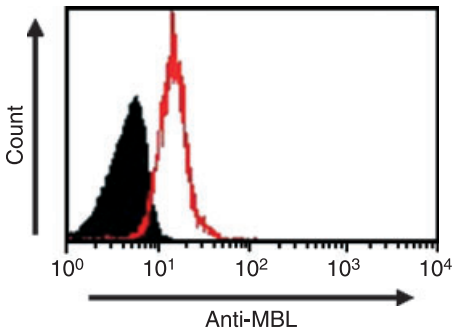

Figure 1. Mannose-binding lectin (MBL) opsonization of zymosan (Zy) detected by flow cytometry. The level of MBL detected on unopsonized Zy (black filled histogram) and MBL-opsonized Zy (open red histogram) following staining with anti-MBL-fluorescein isothiocyanate (FITC) monoclonal antibody (mAb) is shown.

Cell preparation

PBMC, obtained from blood cultured without microbial stimuli, Zy or MBL-Zy, were enriched for blood lineage negative (lin$^-$) cells by magnetic depletion of CD3$^+$, CD14$^+$, CD19$^+$, CD20$^+$, CD56$^+$, CD66$^+$; CD325a$^+$ and CD34$^+$ cells (AutoMACS negative selection; Miltenyi Biotech, North Ryde, NSW, Australia) as described elsewhere.[43] AutoMACS isolated lin$^-$ cells were stained with SAM-FITC, anti-CD15-FITC and anti-CD11c-APC prior to sorting (FACSAria; BDIS, Sydney, Australia). Blood MDCs, representing typically 2–14% of lin$^-$ cells, were sorted as CD11c^{hi} events (> 98% purity). Also, lin$^-$ cells were stained with SAM-FITC, anti-CD15-FITC, anti-CD11c-APC and anti-HLA-DR-PerCP to confirm that gated acquisition of CD11c^{hi} events included exclusively CD11c^{hi} HLA-DR$^+$ events. Fresh blood (200 ml collected in CPDA) was used to prepare naïve CD4$^+$ T cells for allo-T-cell assay. PBMC depleted of CD19$^+$, CD20$^+$, CD11c$^+$, CD14$^+$, CD34$^+$, CD56$^+$, HLA-DR$^+$, CD325a$^+$, CD8$^+$ and CD45RO$^+$ cells were negatively selected. The collected fraction contained > 90% of naïve CD4$^+$ CD45RA$^+$ cells.

Surface phenotype of blood MDCs

To analyse the surface phenotype of blood MDCs, lin$^-$ cells were stained with SAM-FITC, anti-CD15-FITC, anti-CD14-FITC, anti-CD11c-APC and HLA-DR-PerCP

combined with anti-CD40-PE, anti-CD86-PE, anti-CD83-PE, anti-CD62L-PE, anti-CD49d-PE or anti-CXCR4-PE mAbs. Blood MDCs were defined by gated acquisition of HLA-DR$^+$ CD11c^{hi} CD14$^-$ events and the gated MDC population was assessed for expression of costimulatory (CD40 and CD86), activation (CD83), adhesion (CD62L and CD49d) and homing (CXCR4) molecules by flow cytometry. A Becton Dickinson (North Ryde, NSW, Australia) FACS Calibur flow cytometer was used throughout. Analysis was carried out with FCS EXPRESS v3. (De Novo Software, Los Angeles, CA, USA)

Cytokine production by blood MDCs and monocytes

Cytokine production by blood MDCs and monocytes was assayed in whole blood samples, as published elsewhere, with slight modifications.[44] In brief, upon completion of the incubation in 24-well plates, blood samples were stained with anti-CD14-FITC, anti-CD45-PerCP and anti-HLA-DR-APC mAbs followed by red cell lysis with 450 μl of FACS Lysing Solution (BD Biosciences). Cells were permeabilized (FIX&PER kit; CALTAG Laboratories, Invitrogen, Sydney, NSW, Australia) and stained with the anti-IL-6-PE, anti-TNF-α-PE, anti-IL-10-PE or anti-IL-12p40/70-PE mAb. Staining with isotype-matched mouse IgG-PE or rat IgG-PE mAb was used to determine levels of non-specific fluorescence background (< 2% of positive cells). Blood MDCs or monocytes expressing cytokines were analysed within the gated acquisition of blood MDCs (CD45$^+$ HLA-DR$^+$ CD14$^-$ events) or monocytes (CD45$^+$ CD14$^+$ HLA-DR$^+$ events).

Allo-T-cell responses

Sorted blood MDCs (10 000 cells diluted down to 312 cells per well) were cultured with naïve allo-T cells (1×10^5 CD4$^+$ T cells/well) in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all from Invitrogen). Proliferation was measured by adding [^{3}H]thymidine (1 μCi/well) (Amersham) for the last 16 hr of 6-day cultures. Controls consisting of T-cell-only wells, or T cells incubated with lin$^+$ CD11c$^+$ cells substituted for MDCs, demonstrated no proliferation (data not shown). Effector T-cell production was assessed among allo-T cells expanded with 10 ng/ml phorbol myristate acetate and 1 ng/ml ionomycin (both from Sigma), for the last 6 hr of 5-day cultures (DC:T cell ratio 1 : 10). GolgiPlug (BD Biosciences) was added during the last 2 hr to accumulate cytokine protein in the Golgi complex. Cells were stained with anti-CD3-FITC mAb, fixed/permeabilized (FIX&PER kit) and then stained with anti-IFN-γ-PE, anti-IL-2-PE, anti-IL-10-PE or anti-IL-4-PE mAb. Staining with isotype-matched mouse IgG-PE or rat IgG-PE mAb was used to determined levels of non-specific fluorescence background (< 2% of positive cells). Allo-CD3$^+$ T effector cells expressing cytokines were analysed within the gated acquisition of CD3$^+$ events.

Statistical analysis

GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and representations of graphs. As appropriate for the sample size in the study, non-parametric data were compared using the unpaired Mann–Whitney test with 95% confidence intervals (CIs). Paired non-parametric measures were analysed with the Wilcoxon matched pairs test with 95% CIs. Significance was defined as $P < 0{\cdot}05$.

Results

Description of MBL-D and MBL-S individuals

The MBL mannan-binding level, C4 deposition function and *MBL2* genotype were determined for 101 healthy adults recruited for the study. From this cohort, nine MBL-D individuals and seven MBL-S individuals were selected. The *MBL2* genotype, functional mannan-binding MBL level and C4 deposition of these individuals are listed in Table 1.

Zy stimulation induced similar changes in the surface phenotypes of blood MDCs in MBL-D and MBL-S individuals

We stimulated whole blood cultures with Zy or MBL-Zy and assessed the expression of costimulatory (CD40 and CD86), activation (CD83), adhesion (CD62L and CD49d) and homing (CXCR4) molecules on blood MDCs after 6 hr. In MBL-S individuals, stimulation with Zy resulted in significantly decreased proportions of MDCs expressing CD86 and CD49d, and significantly increased proportions of MDCs expressing CD83 (Fig. 2). Similarly, in MBL-D individuals, stimulation with Zy significantly decreased the proportions of blood MDCs expressing CD86 and significantly increased the proportions of MDCs expressing CD83 (Fig. 3; unfilled box and whisker plots). Reduced CD49d expression was evident in Zy-stimulated MDCs from MBL-D individuals, although this did not reach significance. In addition, Zy-stimulated MDCs from both MBL-S and MBL-D individuals had reduced expression of CD62L and CXCR4, although this did not reach significance. In both MBL-S and MBL-D individuals, stimulation with MBL-Zy altered the phenotype of blood MDCs in a similar fashion to Zy stimulation (Fig. 3). These data suggest that MBL deficiency does not play an obvious role in the Zy-induced changes in the surface phenotype of blood MDCs.

Table 1. Characteristics of mannose-binding lectin-sufficient (MBL-S) and mannose-binding lectin-deficient (MBL-D) individuals

MBL2 genotype (abbreviated)	MBL level (μg/ml)	C4 deposition (U C4/μl)
MBL-S		
HYA/HYA (*AA*)	2·88	0·42
HYA/LYA (*AA*)	3·13	0·74
HYA/LYA (*AA*)	1·61	0·52
HYA/LYA (*AA*)	3·66	0·90
LXA/LYA (*AA*)	1·84	0·52
LXA/LYA (*AA*)	1·18	0·36
LXA/LYA (*AA*)	1·85	0·42
MBL-D		
LXA/HYD (*AD*)	0·04	0·07
LXA/LYB (*AB*)	0·00	0·05
LXA/LYB (*AB*)	0·00	0·05
LXA/LYB (*AB*)	0·00	0·15
HYD/HYD (*DD*)	0·00	0·06
HYD/HYD (*DD*)	0·00	0·07
LYB/LYB (*BB*)	0·00	0·09
HYD/LYB (*DB*)	0·00	0·05
HYD/LYB (*DB*)	0·00	0·03

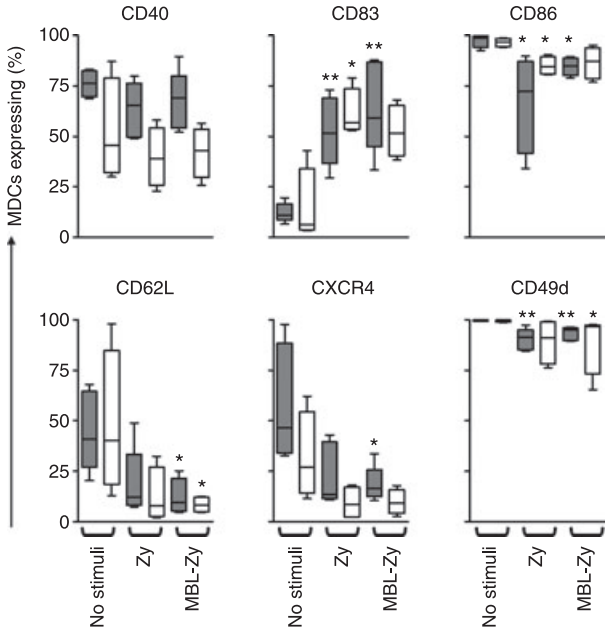

Figure 2. Surface phenotype of blood myeloid dendritic cells (MDCs). Blood MDCs were maintained in whole blood culture with no stimuli, zymosan (Zy) or mannose-binding lectin opsonized Zy (MBL-Zy) for 6 hr, stained with monoclonal antibody (mAb) and analysed for surface phenotype by flow cytometry. The proportions of blood MDCs expressing CD40, CD86, CD83, CD62L, CD49d and chemokine (C-X-C motif) receptor 4 (CXCR4) are indicated in the box and whisker plots. Data are representative of five MBL-sufficient (MBL-S; grey filled) and four MBL-deficient (MBL-D; unfilled) individuals. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$ when compared with MDCs of the same group (MBL-S or MBL-D) maintained with no stimuli.

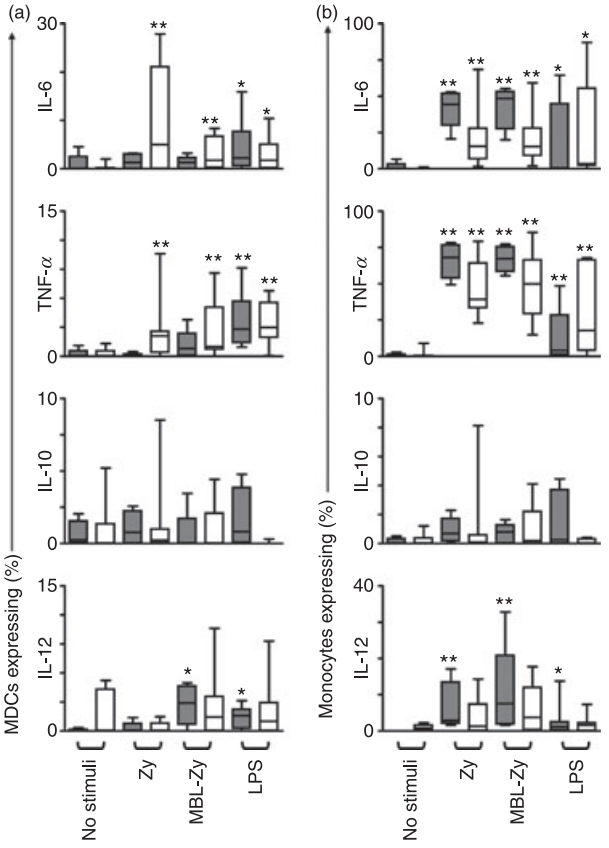

Figure 3. Inflammatory cytokine profiles of blood myeloid dendritic cells (MDCs) and monocytes. Blood MDCs or monocytes were maintained in whole blood culture with no stimuli, zymosan (Zy), mannose-binding lectin opsonized Zy (MBL-Zy) or lipopolysaccharide (LPS) for 6 hr, stained with monoclonal antibody (mAb) and analysed for inflammatory cytokine profile by flow cytometry. The proportions of blood MDCs (a) or monocytes (b) producing interleukin (IL)-6, tumour necrosis factor (TNF)-α, IL-10 or IL-12 are indicated in the box and whisker plots. Data are representative of five MBL-sufficient (MBL-S; grey filled) and nine MBL-deficient (MBL-D; unfilled) individuals. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$ when compared with MDCs or monocytes of the same group (MBL-S or MBL-D) maintained with no stimuli.

Zy stimulation induced proinflammatory cytokine production by blood MDCs in MBL-D individuals

We then proposed that MBL deficiency may influence cytokine production by blood MDCs in response to Zy stimulation. To test this hypothesis, we investigated the production of inflammatory cytokines by blood MDCs and monocytes following Zy and MBL-Zy stimulation and compared these with the inflammatory cytokine profiles induced in the absence of stimulation, and in response to LPS stimulation. In both MBL-S and MBL-D individuals, < 5% of MDCs produced IL-6, TNF-α, IL-10 and IL-12 in the absence of overt pathogen (Fig. 3a). In MBL-S individuals, Zy stimulation did not induce

significant production of IL-6, TNF-α, IL-10 or IL-12 by MDCs compared with that seen in the absence of stimulation. In contrast, Zy stimulation of MDCs from MBL-D individuals significantly increased the proportions of blood MDCs producing IL-6 and TNF-α compared with those detected in the absence of stimulation. However, the proportions of blood MDCs producing IL-10 and IL-12 were not changed in MBL-D individuals following Zy stimulation (Fig. 3a).

In MBL-S individuals, MBL-Zy stimulation induced a similar IL-6, IL-10 and TNF-α cytokine response to blood MDCs that were stimulated with Zy. Significantly increased IL-12 production was observed in MBL-Zy-stimulated MDCs from MBL-S individuals (Fig. 3a). In contrast, MBL-Zy stimulation of MDCs from MBL-D individuals significantly reduced the proportion of blood MDCs expressing IL-6, but not TNF-α (Figs 3a and 4a). We then tested whether supplementing the MBL-D blood culture with MBL would alter the cytokine response to Zy by MBL-D individuals. Adding hpMBL to the culture with Zy also reduced the proportions of blood MDCs producing IL-6 when compared with those induced by Zy stimulation (Fig. 4b), suggesting that pre-opsonization was not a requirement for the MBL-mediated influence on IL-6.

Clear differences in inflammatory cytokine production between blood MDCs of MBL-D and MBL-S individuals induced by Zy and MBL-Zy stimulation were not manifest after LPS stimulation (Fig. 3a). In contrast to blood MDCs, inflammatory cytokine production by monocytes induced by Zy, MBL-Zy or LPS stimulation was similar in MBL-D and MBL-S individuals (Fig. 3b). These data suggested that MBL deficiency results in enhanced proinflammatory cytokine production by blood MDCs, but not monocytes, in response to microbial stimulation, and that MBL may have a regulatory role in IL-6 production by blood MDCs in response to pathogenic stimulation.

Blood MDCs of MBL-deficient individuals stimulated with Zy are poor inducers of allo-T effector cells

We further investigated whether different production of proinflammatory cytokines by blood MDCs of MBL-D individuals in response to Zy may have an impact on T-cell responses. Blood MDCs, isolated from non-stimulated, Zy- or MBL-Zy-stimulated whole blood cultures, were assessed for their capacity to induce proliferation and effector differentiation of naïve allo-T cells. Blood MDCs from either MBL-D or MBL-S individuals, following stimulation with Zy or MBL-Zy, showed an increased capacity to induce proliferation of allo-T cells (Fig. 5).

Generally, blood MDCs of MBL-D individuals stimulated with either Zy or MBL-Zy were poor inducers of allo-T effector cells, compared with non-stimulated blood MDCs (Fig. 6). There was a trend for lower proportions

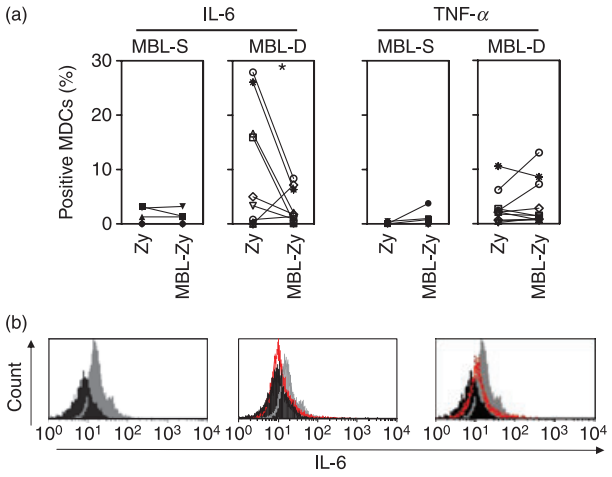

Figure 4. Comparison of zymosan (Zy)- and mannose-binding lectin opsonized Zy (MBL-Zy)-mediated interleukin (IL)-6 and tumour necrosis factor (TNF)-α production in MBL-sufficient (MBL-S) and MBL-deficient (MBL-D) individuals. (a) Proportions of blood myeloid dendritic cells (MDCs) in MBL-S ($n = 5$) and MBL-D ($n = 9$) individuals producing IL-6 and TNF-α in the presence of Zy or MBL-Zy. Paired data are connected by a line. *$P < 0{\cdot}05$ denotes significant differences when comparing Zy versus MBL-Zy stimulation (for the six donors with detectable IL-6 following Zy stimulation). (b) Representative histograms showing proportions of blood MDCs producing IL-6 in MBL-D individuals in the presence of Zy, MBL-Zy and hpMBL (5 μg/ml) in culture with Zy. Left histogram: proportions of blood MDCs producing IL-6 in response to Zy (grey filled) and the isotype control (black filled). Middle histogram: proportions of blood MDCs producing IL-6 in response to Zy (grey filled) and MBL-Zy (red line), and the isotype control (black filled). Right histogram: proportions of blood MDCs producing IL-6 in response to Zy (grey filled) and hpMBL in culture with Zy (red line), and the isotype control (black filled).

of allo-T effector cells expressing IFN-γ, IL-10 and IL-4 in cultures with MDCs stimulated with Zy or MBL-Zy, compared with cultures with non-stimulated blood MDCs. Only MDCs stimulated with MBL-Zy appeared to have the capacity to induce higher proportions of allo-T cells producing IL-2, compared with non-stimulated blood MDCs (Fig. 6). In contrast, MDCs from MBL-S individuals stimulated with either Zy or MBL-Zy induced greater proportions of allo-T effector cells expressing IL-2 and IL-10, compared with non-stimulated blood MDCs (Fig. 6). These data suggest that MBL deficiency may account for the reduced capacity of blood MDCs to induce effector T-cell responses following encounter of pathogen containing MBL ligands.

Discussion

MBL is a component of our innate immune system, and a lack of functional MBL to bind pathogens and activate the complement cascade may have important

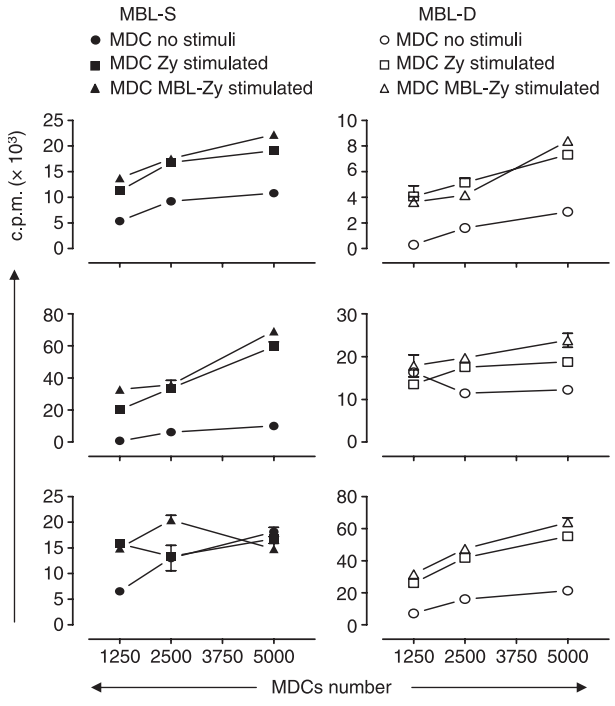

Figure 5. Allo-T-cell proliferation induced by blood myeloid dendritic cells (MDCs). Blood MDC maintained in whole blood culture with no stimuli, zymosan, mannose-binding lectin (MBL)/zymosan for 6 hr, and then cultured with naïve allo-CD4$^+$ T cells (1×10^5 CD4$^+$ T cells/well) in different MDC/T cell ratios (1 : 10–1 : 800) for 6 days. Proliferation of naïve allo-CD4$^+$ T cells was measured by [^{3}H] thymidine incorporation. The data are displayed as the mean count per minute (c.p.m.) values ± SEM of triplicate measurements of three representative MBL-deficient and MBL-sufficient individuals. c.p.m., counts per minute.

consequences for the health of an individual. Indeed, MBL deficiency has been associated with a number of important infectious,[16–20] inflammatory[21–23] and autoimmune disease states[45] in humans. However, despite a large number of clinical studies indentifying the importance of MBL in relation to disease, further insights into the mechanisms of MBL deficiency and the apparently reduced ability of MBL-D individuals to respond to infection are lacking. Previous studies in this regard have focused on the interactions between MBL and monocytes,[36] macrophages[36] and neutrophils;[38] particularly on the role of MBL in opsonization and phagocytosis. To date, the effects of MBL deficiency on blood DC activation and the subsequent T-cell responses that would determine an individual's ability to respond appropriately to an infectious episode have not been investigated.

In this study we utilized a whole blood assay to compare the MBL-S and MBL-D phenotypes and functions of blood MDCs in the absence of overt pathogen and following stimulation with microbial components LPS and Zy. In addition, we investigated the potential for hpMBL to influence DC function, as either MBL-opsonized Zy or hpMBL in culture with Zy.

Further to surface phenotype analyses, the data presented indicate that Zy induced IL-6 and TNF-α production by blood MDCs from MBL-D individuals but not by MDCs from MBL-S individuals, suggesting a proinflammatory phenotype of MDCs in MBL-D compared with MBL-S individuals. Moreover, we demonstrated that the addition of MBL in the form of MBL-opsonized Zy (MBL-Zy) or hpMBL spiked into the whole blood culture containing Zy reduced IL-6 production by blood MDCs in MBL-D individuals. Taken together, these data suggest a specific interaction between blood MDCs and Zy in MBL-D individuals, as Zy induced similar IL-6 production by monocytes in both MBL-D and MBL-S individuals.

As a proinflammatory cytokine, IL-6 plays an important role in the immune response. IL-6 regulates the expression of acute phase response proteins,[46] affects DC maturation and survival[47] and has been reported to play a role in T-cell differentiation.[48] In the clinical setting, high IL-6 levels are associated with poor prognosis and poor outcome in sepsis and systemic inflammatory response syndrome.[49] Increased IL-6 produced by MDCs in MBL-D individuals may have important clinical

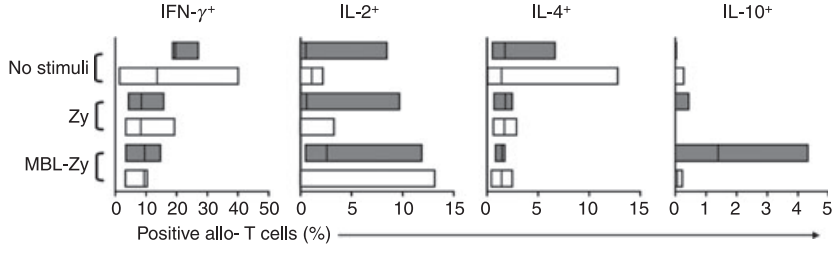

Figure 6. Allogeneic (allo)-T cell effectors induced by blood myeloid dendritic cells (MDCs). Blood MDCs were maintained in whole blood culture with no stimuli, zymosan (Zy) or mannose-binding lectin (MBL)-Zy for 6 hr, and then cultured with naïve allo-CD4$^+$ T cells (1×10^5 CD4$^+$ T cells/well) at an MDC:T-cell ratio of 1 : 10 for 5 days. The proportions of allo-CD4$^+$ T cells producing interferon (IFN)-γ, interleukin (IL)-2, IL-10 and IL-4 are indicated. The data are displayed as low–high bar graphs with the line at the mean for four MBL-sufficient (MBL-S; grey filled) and four MBL-deficient (MBL-D; unfilled) individuals.

implications and suggests a mechanism by which MBL-D individuals may suffer from a reduced ability to respond to pathogen.

In addition to the clear effect of MBL-D on MDC IL-6 production, we found some evidence that MBL deficiency may influence IL-12 responses. In MBL-S individuals both MBL-Zy and LPS significantly enhanced the proportions of MDCs producing IL-12. While increases were observed in IL-12 production in MBL-D individuals with these same stimuli, they did not reach significance. The influence of MBL on IL-12 invites further investigation. Beyond the potential differences in IL-12 production, the data presented suggest minimal differences in the ability of blood MDCs and monocytes from MBL-D and MBL-S individuals to respond to soluble LPS stimulation. This was not surprising as soluble LPS is not generally recognized as an MBL ligand. Using particulate Zy as a stimulus for blood MDCs and monocytes may involve different recognition mechanisms, compared with LPS recognition. In particulate form, Zy engages PRRs and can be phagocytosed, leading to engagement of different immune recognition mechanisms.[50] Recently, MBL has been reported as influencing Toll-like receptor 2 (TLR2) and TLR6 responses.[52] TLR2 is a PRR for Zy and data from the current study support a role for MBL as a regulator of inflammatory responses through TLR2.

Despite differences in cytokine profile between blood MDCs from MBL-D and MBL-S individuals in response to Zy stimulation, they demonstrated a similar capacity to induce proliferation of allo-T cells in the mixed lymphocyte response (MLR) assay. However, MLR assays involve different donor–responder pairing, and this experimental limitation must be taken into account when comparing the level of allo-T-cell proliferation induced by blood MDCs from MBL-D individuals versus blood MDCs from MBL-S individuals.

Despite a similar capacity to induce proliferation of allo-T cells, blood MDCs from MBL-D individuals stimulated with Zy induced fewer IL-2^+ and IL-10^+ effector T cells compared with MDCs from MBL-S individuals. This raises the possibility that the pattern of cytokine (e.g. IL-6) production induced by Zy and MBL-Zy stimulation of blood MDCs in MBL-D individuals may account for their limited ability to induce effector T cells. These data suggested that MBL-D individuals (after encounter with MBL-ligand containing pathogenic stimuli) may have difficulties in mounting IL-2^+ and IL-10^+ effector T-cell responses. An increase in allo-T cells was observed in MBL-D individuals following stimulation with MBL-Zy. Overall, this suggested that a lack of MBL can account for reduced effector T-cell responses and that this can be abrogated by addition of MBL (in the case of IL-2). MBL deficiency therefore appears to have an influence on T effector aspects of immune responses.

We carried out preliminary experiments to address differences in T regulatory cell (Treg) numbers in the allogeneic MLR induced by MDCs from MBL-S and MBL-D individuals. There was a substantial individual-to-individual variation in the numbers of Fox $P3^+$ $CD25^+$ Treg in the MBL-S individuals measured and the MBL-D measurement fell within this range (data not shown). In these preliminary experiments, no difference in Treg numbers attributable to MBL deficiency was observed.

Interestingly, we found that MBL deficiency did not adversely influence blood MDC surface phenotype for the range of molecules studied. Mechanisms involving effects of MBL on pathogen recognition molecules (e.g. TLR2), collectin receptors, phagocytosis, and resulting signaling and cytokine responses rather than costimulation may drive the altered MDC function in MBL-D individuals.

The possibility that the endogenous MBL, present in MBL-S individuals, competed with MBL on hpMBL-opsonized Zy in the whole blood model requires consideration. In the experiments in this study, Zy was opsonized with 5 μg/ml hpMBL, which is at the high end of MBL levels seen in the population. In all MBL-S individuals included in this study, the range of levels was considerably below 5 μg/ml (see Table 1) and thus endogenous MBL was unlikely to compete with MBL already bound to the zymosan. It is possible that the results obtained for MBL-S individuals in the MBL-Zy stimulation would have an additive effect of the endogenous MBL, further opsonizing the Zy.

In this study, we have utilized a whole blood assay to model infection in healthy MBL-D and MBL-S individuals. The data presented suggest an additional mechanism for the increased frequency and severity of infection seen in MBL-D individuals. Based on this premise, it would be warranted to carry out similar experiments on a group of MBL-D patients (e.g. post bone marrow transplantation). In such settings, the differences between MBL-D and MBL-S individuals may be greater. Such findings could be usefully translated to immunotherapy supporting MBL supplementation as a way to improve immune responses in MBL-D individuals.

In conclusion, the novel findings in this paper reveal that MBL deficiency is associated with unique functional characteristics of pathogen-stimulated blood MDCs manifested through increased production of IL-6, combined with a poor capacity to induce effector allo-T-cell responses. In MBL-D individuals, these functional features of blood MDCs may arise during an infectious episode and may adversely influence their ability to mount an effective immune response. Furthermore, the *in vitro* addition of MBL reversed these altered phenotypes, supporting the introduction of MBL-based immunotherapy and suggesting an additional mechanism of action for this soluble immune pathogen recognition molecule.

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Disclosures

The authors have no conflicts of interest, or financial conflicts to disclose.

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