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Significance of monocytic cytokines at single cell level for the immune responsiveness in renal transplant recipients

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Abstract Cytokine dysregulation is an important factor underlying the immune unresponsiveness to hepatitis B vaccination (HBV) in renal transplant recipients. This study investigated the relationship between monocyte-derived interleukin-6 (IL-6) and interleukin-10 (IL-10) production and the immune responsiveness using flow cytometry (cytoflow) after whole blood culture. According to their previous response to hepatitis B vaccination, 40 renal transplant recipients were divided into two groups of 20 patients. The percentage of CD14+ monocytes stained positive for intracellular IL-6 or IL-10 was measured using flow cytometry after 4 and 20 h of whole blood culture with lipopolysaccharide stimulation. The percentage of CD14+/IL-6+ cells after incubation in vitro for 4 h was lower in the responders compared to the non-responders and controls (27.15 ± 8.93 vs 35.47 ± 9.95, $P = \text{NS}$; and 37.06 ± 10.89, $P < 0.05$ respectively). The staining intensity of IL-6 at 4 h for responders was also significantly reduced. At 20 h, there were a significantly higher percentage of CD14+/IL-10+ positive cells in the responders compared to the non-responders (41.87 ± 18.39 vs 27.55 ± 17.25, $P < 0.05$). These results indicate that alteration of intracellular cytokine profile in activated monocytes distinguishes the HBV vaccination responders from the non-responders among renal transplant recipients. The capacity to upregulate monocyte IL-10 production in this subset of patients may modulate the immune responsiveness and effectively assists in mounting a positive response to HBV vaccination.

Keywords Whole blood · Monocyte · Intracellular · Cytokines · Interleukin-6 · Interleukin-10

Abbreviations *CSIF* Cytokine synthesis inhibitory factor · *HBV* Hepatitis B vaccination · *HD* Haemodialysis · *IL-6* Interleukin-6 · *IL-10* Interleukin-10 · *LPS* Lipopolysaccharide · *RT* Room temperature · *PBS* Phosphate-buffered saline · *RTR* Renal transplant recipients · TNF-α Tumor necrosis factor-alpha

Introduction

Anti-rejection therapy after renal transplantation often induces a state of immunodeficiency in kidney transplant recipients. Leukocytes of various lineages are suppressed functionally in multiple aspects. These are manifested clinically as increased susceptibility to infection, higher incidence of malignancy, and diminished responsiveness to hepatitis B (HBV) vaccination [13]. In a 12-month follow-up study, Jacobson showed

a cumulative response rate of only 17.6% after standard HBV vaccination in renal transplant recipients (RTR) [13]. The sero-response rate was much lower than that of haemodialysis (HD) patients (58–70%) [2, 11, 12, 20] and immunocompetent individuals (>95%). Moreover, among RTR who mounted an antibody response, anti-HBs titers were generally low and sometimes declining rapidly. The reduced sero-response rate was not related to alterations in T4/T8 ratio. The earliest responders actually had the lowest T4/T8 ratio [13]. In patients on HD, the antibody response against HBV vaccination had been shown to correlate well with cellular immune functions, especially the activation capabilities of antigen-presenting cells [8, 16]. In this regard, a defect in the co-stimulatory function of monocytes/macrophages was pathogenetically linked to the uraemic state [11]. Alternatively, dysregulated production of monocyte-derived cytokines interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) have been implicated in the suboptimal response of HBV vaccination in dialysis patients [9]. High level of IL-6 correlates with impaired immune status, and this defect could be corrected by upregulation of IL-10 secretion in monocytes [9]. In RTR, alterations of cytokine profile are more complicated as a result of the immunosuppressive therapy. The intricate relationship between monocyte-derived cytokine production and the response to HBV vaccination remains to be explored.

In this study, we investigated the relationship between the sero-response of RTR to HBV vaccination and monocytic cytokine production. IL-6 and IL-10 were detected both in serum and at single cell level using three-color immunofluorescent staining following whole-blood culture. We detected a significant difference in the stimulated cytokine production between responders and non-responders.

Patients and methods

Patients

Forty RTR were recruited for the study. Eligibility criteria included renal transplantation for more than 2 years and stable serum creatinine of less than 200 μmol/l. All patients received cyclosporine, prednisolone and azathioprine as their immunosuppressive treatment. Patients with documented acute rejection or active infection within 3 months prior to the study were excluded. Eligible patients were divided into two groups of 20 according to their previous response to HBV vaccination, using Engerix B (Merck Sharp & Dohme, Whitehouse Station, NJ, USA) 40 μg given at 0, 1, 6 months. The antibodies to HBs in serum were detected using a sandwich ELISA (VIDAS Anti-HBs Total, Vitek Immuno Diagnostic Assay System, Barcelona, Spain) Positive sero-response was defined as anti-HBs concentration equal or greater than 10 mIU/ml [6, 15, 23]. 19 age-matched healthy volunteers were included as control.

Cells and cell culture

One milliliter of peripheral blood was collected from the patients and controls in plastic bottles containing 50U lithium heparin before their morning medications. 100 μl whole blood was added to an equal volume of RPMI (Sigma, St. Louis, MO, USA) containing 2.0 μg/ml lipopolysaccharide (LPS) (E. coli 0111; B4; Sigma, St. Louis, MO, USA) in the presence of 4 μM monensin (Golgistop®, Pharmingen, San Diego, CA, USA). The cultures were carried out in 12×75mm polypropylene round-bottomed tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for 4 h or 20 h at 37°C and 5% CO_2.

Monoclonal antibodies

The following monoclonal antibodies were used. They were phycoerythrin/cyanin 5 (PC5)-labeled anti-CD14 (mouse IgG2a, Immunotech, Marseilles, France), FITC-labeled anti-IL-6 (rat IgG1, Pharmingen, San Diego, CA, USA) and PE-labeled anti-IL-10 (rat IgG1, Pharmingen, San Diego, CA, USA). Mouse and rat IgG (Sigma, St. Louis, MO, USA) were used to block the Fc receptors on human cells in order to reduce non-specific immunofluorescent staining of test antibodies. FITC- and PE-conjugated rat IgG1 isotype antibodies were used to control for background staining.

Cytokine measurement by flow cytometry ("cytoflow technique")

Fixation, permeabilization and staining were performed with IntraPrep fixation and permeabilization reagents (Immunotech, Marseilles, France) following manufacturer's protocol. Briefly, 200 μl of IntraPrep Reagent 1 (5.5% formaldehyde) was added to each tube and incubated for 15 min at room temperature (RT). After vigorous vortex mixing, cells were washed with 4 ml phosphate-buffered saline (PBS) and the supernatant was discarded by aspiration after centrifugation (300 *g*, 5 min). The cells were then incubated with 100 μl of IntraPrep Reagent 2 (saponin-based permeabilizing and lysing medium) for 5 min at RT. The red cells were lysed using a platform-appropriate lysing solution and the remaining cells were washed with 2 ml PBS containing 0.02% sodium azide (Sigma, St. Louis, MO, USA) and 0.2% bovine serum albumin (Sigma, St. Louis, MO, USA). The cells were pelleted at 300 *g* for 5 min.

The leukocyte Fc receptors were pre-blocked by incubation with 1 μg mouse IgG and 1μg rat IgG for 20 min at RT. After incubation with the anti-cytokines and anti-CD14 antibodies at a concentration of 0.25 μg/ml for 20 min at RT in the dark, they were washed twice in PBS and then resuspended in 500 μl PBS containing 0.5% formaldehyde. To determine the percentage of dead cells and the effects of monensin on surface antigens, some cultured cells were not fixed, but washed in Hank's buffered salt solution (Sigma, St. Louis, MO, USA) containing 0.1% sodium azide. The specimens were stored at 4°C and analyzed with a FACS cytometer.

The stained cells were analyzed using a Coulter EPICS XL analyzer (Coulter electronic, Miami, FL, USA). A minimum of 5,000 fixed cells for each sample were analyzed. The percentage of cells positive for CD14 and the cytokines staining was calculated against a negative control, with the cytokine-specific antibodies omitted during the staining procedure. Cutoff markers were set individually for each measurement to give a 4% reading for the negative control. The fluorescence intensity of individual cell, which was related to the amount of cytokine produced per cell, was registered. The mean fluorescence intensity (MFI) for a cytokine was calculated as the geometric mean of all cells above cutoff.

Statistical analyses

Data management and statistical analyses were performed using Prism V3.00 statistical software (Graphpad Software, San Diego,

CA, USA). All data were given as mean ± SD. Significance of differences between patients and control subjects was calculated by Mann-Whitney test, and correlations were detected by Spearman's rank correlation analysis. All tests were considered significant if $P < 0.05$.

Results

The demographic data of the patients are shown in Table 1. There was no difference in the sex ratio, age, duration of transplantation and underlying renal diseases between control, responders and non-responders.

The total amount and percentages (in bracket) of CD14+ monocytes in the unstimulated whole blood samples of normal controls, responders and non-responders were 0.39 ± 0.13×109/l (5.2 ± 0.6%), 0.36 ± 0.16×109/l (4.9 ± 0.5%) and 0.35 ± 0.15×109/l (4.8 ± 0.5%), respectively. There was no difference between the three groups at baseline ($P > 0.05$) and after incubation with LPS (data not shown). The percentage of cells stained positive for IL-6 and IL-10 and their fluorescence intensity are shown in Table 2. The percentage of CD14+/IL-6+ cells after incubation in vitro for 4 h was lower in the responders than in the non-responders and controls (27.15 ± 8.93 vs 35.47 ± 9.95, P = NS, and 37.06 ± 10.89, $P < 0.05$ respectively) (Fig. 1a). The percentage of CD14+/IL-10+ monocytes, however, was not different among the 3 groups (Fig. 1b) (responders 20.71 ± 14.71, non-responders 19.95 ± 14.15 and control 16.60 ± 8.16, P = NS). The staining intensity of IL-6 at 4 h for responders was significantly lower than that of control and non-responder (Fig. 1c), whilst for IL-10, no difference was detected (Fig. 1d). At 20 h, the percentage of CD14+/IL-6+ cells was lower in the responders, though it was not statistically significant (Fig. 2a). However, there was a significantly higher percentage of CD14+/IL-10+ positive cells in the responders compared to the non-responders (41.87 ± 18.39 vs 27.55 ± 17.25, $P < 0.05$) (Fig. 2b). At 20 h, the staining intensity for IL-6 and IL-10 did not show any significant difference among the three groups (Fig. 2c, d).

Table 1. Patient characteristics

Characteristic	Control	Responder	Non-responder
Age (years)	45.36 ± 7.35	44.50 ± 8.56	43.90 ± 11.88
Gender (M:F)	10:9	11:9	9:11
Duration of transplant (months)	–	76.25 ± 44.38	88.83 ± 78.66
Serum creatinine (μmol/l)	89 ± 11.7	137.15 ± 47.33	140.85 ± 51.06
Type of transplant			
Cadaveric:living-related	–	11:9	14:6
Immunosuppressives (mg/day)			
Prednisolone	–	6.38 ± 2.63	6.81 ± 1.88
Cyclosporin A	–	172.50 ± 49.97	163.75 ± 67.61
Azathioprine	–	30.00 ± 29.91	28.75 ± 31.70
Underlying renal disease			
Alport syndrome	–	–	1
Chronic PN	–	1	1
DM	–	2	2
IgA nephropathy	–	2	–
Polycystic kidney disease	–	2	–
SLE	–	2	1
TB	–	1	–
Unknown	–	10	15

Table 2. Production of IL-6 and IL-10 by monocytes from renal transplant recipients (responders and non-responders to HBV vaccination) and healthy control

Characteristic	Control	Non-responder	Responder
% IL-6$^+$ cells at 4 h	37.06 ± 10.89	35.47 ± 9.95	27.15 ± 8.93[a]
% IL-10$^+$ cells at 4 h	16.6 ± 8.16	19.95 ± 14.15	20.71 ± 14.71
MFI of IL-6 at 4 h	2.05 ± 0.64[d]	1.63 ± 0.14[c]	1.52 ± 0.08[b]
MFI of IL-10 at 4 h	1.97 ± 0.65	1.56 ± 0.33	1.68 ± 0.55
% IL-6$^+$ cells at 20 h	45.62 ± 11.14	39.92 ± 14.54	35.26 ± 15.43
% IL-10$^+$ cells at 20 h	34.74 ± 16.23	27.55 ± 17.25[c]	41.87 ± 18.39
MFI of IL-6 at 20 h	3.74 ± 1.05	3.13 ± 0.95	3.01 ± 0.96
MFI of IL-10 at 20 h	2.23 ± 0.89	1.67 ± 0.43	2.00 ± 1.03

[a] $P < 0.05$, responder vs. control
[b] $P < 0.001$, responder vs. control
[c] $P < 0.05$, responder vs. non-responder
[d] $P < 0.01$, non-responder vs. control

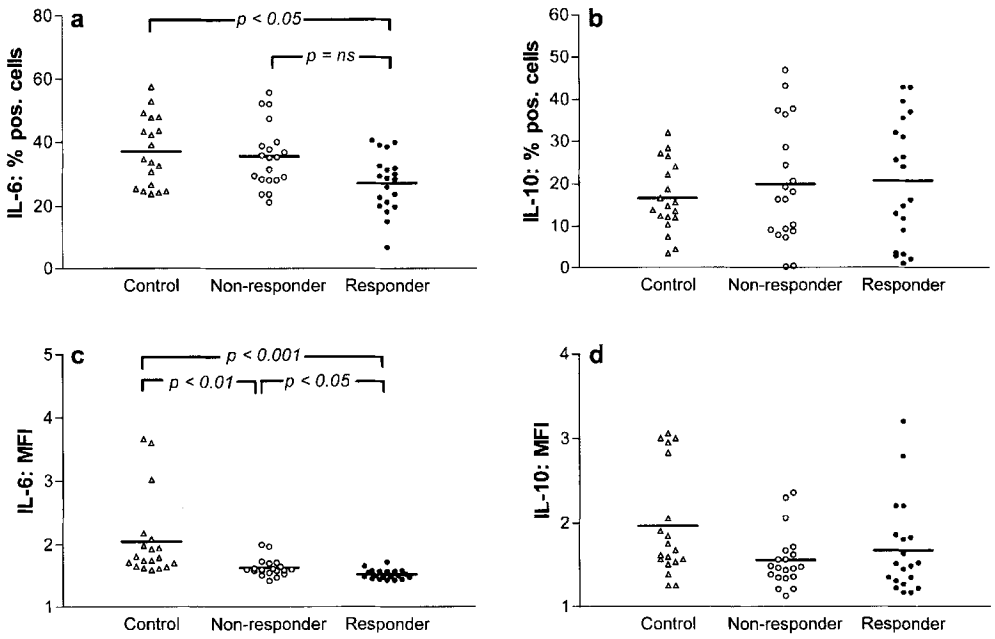

Fig. 1a–d. Intracellular monocytic IL-6 and IL-10 production after stimulation by LPS for 4 h in vitro. **a** and **b** show the percentage of cells positive for IL-6 (**a**) and IL-10 (**b**). **c** and **d** give the MFI as a parameter for the amount of the respective cytokine per cell

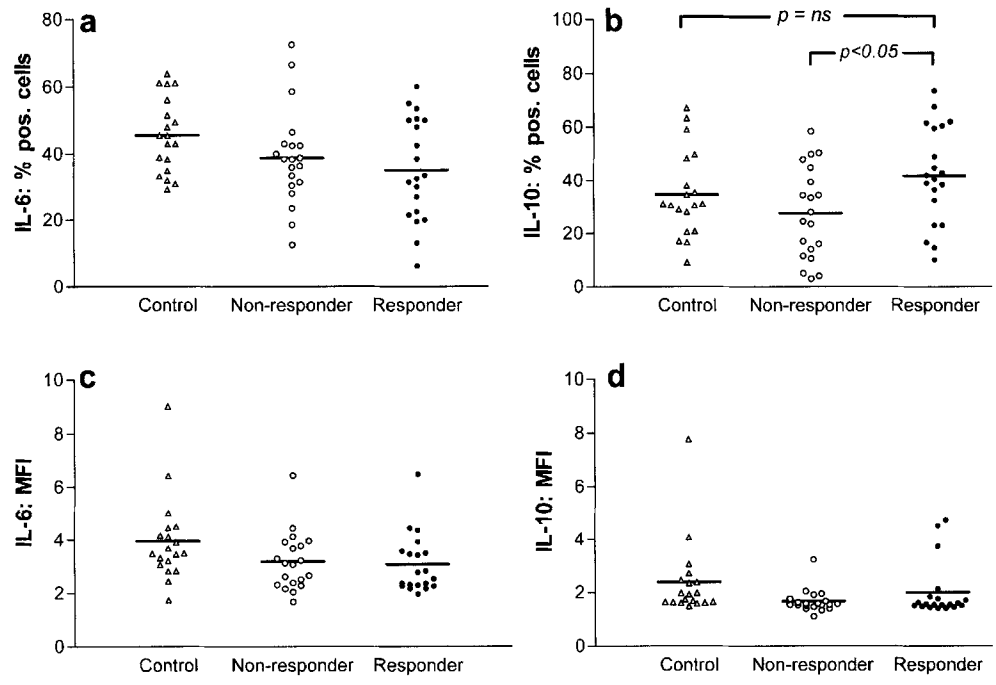

Fig. 2a–d. Intracellular monocytic IL-6 and IL-10 production after stimulation by LPS for 20 h in vitro. **a** and **b** give the percentage of cells positive for IL-6 (**a**) and IL-10 (**b**). **c** and **d** give the MFI as a parameter for the amount of the respective cytokine per cell

Clinical correlation of cytokine production and patient characteristics

There was no association between the percentage of IL-6+ or IL-10+ monocytes with age, duration of transplantation, and dose of immunosuppressives, after both 4 h and 20 h of activation (data not shown).

Discussion

The acquired immunity is a diverse response and involves the interaction between T cells, B cells, monocytes and their cytokines. Previous investigations had shown a good correlation between reduced proliferation of

peripheral blood mononuclear cells, impaired secretion of interleukin-2, and enhanced expression of IL-2 receptor on resting T cells with the clinical sequelae of immunodepression [9]. The response to HBV vaccination in dialysis patients has been regarded as a good predictor of immunocompetence. In this study, two groups of renal transplant recipients (RTR), characterized by a differential anti-HBs sero-response, were investigated. We examined the relationship between monocyte-derived IL-6 and IL-10 production and the immune responsiveness using flow cytometry (cytoflow) after whole blood culture with lipopolysaccharide stimulation. Patients with previous positive sero-response to HBV vaccination showed a significant depression of IL-6 production at 4 h. The fluorescence intensity was lower than that of normal control and non-responders. IL-10 has a longer latency of activation, and the difference between the three groups was only evident after 20 h of incubation. An increased percentage of IL-10+/CD14+ monocytes was detected in the responders. IL-6 production was also lower in the responders at 20 h, though it was not statistically significant. Parallel determination of the culture supernatant was not performed as the number of monocytes varied with different samples.

Overproduction of IL-6 in hemodialysis patients is associated with an immunodeficient state [9]. By classifying the patients into responders and non-responders to hepatitis B vaccination, Girndt et al. showed a strong correlation between LPS-stimulated IL-6 overproduction and the clinical state of immune unresponsiveness [9]. Though our results do not totally concur with the findings in HD patients, the capacity for IL-6 production in monocytes appears to correlate with the degree of immune anergy. IL-10 inhibits cytokine synthesis by macrophage [7] and down-regulates IL-6 overproduction [9]. Thus, upregulation of monocyte-derived IL-10 production in the responder group may effectively enhance the response to HBV vaccination. This discrepancy may also be ascribed to the effect of immunosuppressants on monocytic cytokine production [18, 22]. It is, therefore, proposed that the cytokine balance, rather than the absolute values, is more important in determining the immune responsiveness. Evidently, other cytokine dysregulations may also play an important part in determining the vaccination response.

IL-10, originally referred to as cytokine synthesis inhibitory factor (CSIF) [17], is an interesting candidate for the regulation of monokine secretion. It is produced by a number of different cells including monocytes, B cells, T cells, [7] and exerts its action mainly on the antigen presenting cells. At low concentrations, monokine production is downregulated. At higher concentration, the antigen presenting function and class II molecule expression are inhibited as well [7]. IL-10 is induced in monocytes by LPS at the same concentration that leads to the secretion of IL-1, IL-6 and TNF-α. Activated monocytes/macrophages produce IL-6 and IL-12, which induce IL-10 production by T cells, and TNF-α enhances IL-10 production by monocytes/macrophages [21]. IL-10, in turn, inhibits IL-6, IL-12, TNFα and its own production. This sequential production of IL-6, IL-12, TNF-α and IL-10 constitutes a cascade of cytokines that may act in an autoregulatory fashion resulting in the development of an appropriate immune system [4]. Impaired production or over-production of any of these cytokines may skew the immune response, resulting in either a defect or normalization of the immune response. The reciprocal changes of IL-6 and IL-10, although not proving a causal relationship, indicates that elevation of IL-10 might be a regulatory mechanism to overcome the immune anergy of RTR to HBV vaccination. Nevertheless, the lack of difference in the cytokine profile in non-responders and normal controls showed that the cytokine producing capacity of CD14+ monocytes may still be intact in RTR.

Measurement of plasma cytokine levels were of limited value [1, 3]. In our study, the serum cytokine levels were generally low and clustered at the lower detection range of the commercial ELISA. Since cytokine production by normal resting cells is minimal, one has to use a strong stimulus, such as LPS, to demonstrate the potential of each cell to synthesize cytokines. The production of cytokines by activated peripheral blood mononuclear cells has been studied at the protein level using bioassays and monoclonal antibody techniques, and at the mRNA level. The use of such bulk-release detection methods often assumes that, under any condition, cells of a given phenotype show identical or similar levels of cytokine secreting behavior. However, such techniques can yield ambiguous results since it is often difficult to ensure that a given cell population is free of contaminating cells which may secrete the same cytokine. Moreover, since cytokines act as mediators of cell-cell communication, relevance should be assigned to the local production of cytokines by individual cell populations. Thus, cytokine measurement in culture supernatants can only give cumulative information over cell number and time. An ideal technique should identify producer cells by cell surface markers and by cytokines simultaneously. Intracytoplasmic staining of cytokines and analysis by flow cytometry seems to fulfill these requirements. Also, with the use of monensin, which inhibits intracellular cytokine traffic pathways and release, the fluorescent signal is further enhanced. The "cytoflow technique" was initially described by Jung et al. [14] in lymphocytes. Girndt [10] adapted the techniques to monocytes and concluded in HD patients that overproduction of IL-6 results, at least in part, from a loss of specificity in regulatory monocytes. However, De Groot et al. [5] clearly demonstrated that Ficoll density gradient centrifugation may affect cell activation status, resulting in considerable alteration of cytokine

profile. Whole blood culture, which contains a physiological level of regulatory cytokines may, therefore, more closely approximate circulating cells in vivo and reflect disease state alterations [19].

Despite the seemingly large overlap in the data presented, the stimulated monocytic cytokine profiles still display a statistically significant difference between responders and non-responders. Larger numbers of patients, and, as a control group,preferably patients with a renal failure, would be helpful in confirming the relationship of the various cytokines in the immune responsiveness in RTR.

In conclusion, we have shown that cytokine detection using cytoflow technique in whole blood culture is a superior method of investigating cytokine production in leukocytes. In RTRs who responded to HBV vaccination, the alteration in cytokine profile distinguishes them from the non-responders. The capacity to upregulate monocyte IL-10 production in this subset of patients may modulate the immune responsiveness and effectively assists in mounting a positive response to HBV vaccination. Profiting from this technique, additional studies will help to elucidate the complex relationship between cytokine production and immune reactivity in RTR.

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