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Monocyte B7 and Sialyl Lewis X modulates the efficacy of IL-10 down-regulation of LPS-induced monocyte tissue factor in whole blood

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Summary. Recent studies have investigated the use of anti-inflammatory cytokine, interleukin 10 (IL-10) to control the development of disseminated intravascular coagulation (DIC) in sepsis by down-regulation of monocyte tissue factor (MTF) induced by lipopolysaccharide (LPS) in the initial phase of the disease. *In vitro* and *in vivo* human studies have shown that a minimal (<1 h) delay in IL-10 treatment significantly reduces the cytokines ability to inhibit LPS-induced MTF expression and the end products of coagulation. In this whole blood *in vitro* study we investigated the role of lymphocyte and platelet interactions with monocytes to up-regulate MTF expression in the presence of IL-10 in the initial phase of exposure to LPS. Individual blockade of monocyte B7 or platelet P-selectin significantly (35%) reduced MTF expression ($P<0{\cdot}05$). IL-10 showed a dose-dependent inhibition of LPS (0·1 μg/ml) induced MTF expression, with 56% inhibition at 1 ng/ml, maximizing at 5 ng/ml IL-10 (75%; $P<0{\cdot}05$). Simultaneous exposure to LPS and IL-10 (1 ng/ml) or addition of IL-10 1 h after LPS, with individual B7 and P-selectin blockade significantly enhanced the inhibition of MTF expression by IL-10 ($P<0{\cdot}05$). We conclude that the efficacy of IL-10 to control DIC could be enhanced by a simultaneous B7 and P-selectin blockade.

Keywords: IL-10, tissue factor, B7, Sialyl Lewis X, LPS.

In the initial hyperinflammatory phase of sepsis, induction of MTF by LPS results in the development of DIC by activation of factor VII followed by factor X and IX (Rivers *et al*, 1992). However, DIC surviving patients then become immunosuppressed or 'immunoparalysed' (Volk *et al*, 1996; Astiz *et al*, 1996), which is characterized by the presence of unresponsive monocytes, T cells, NK cells, and an increased rate of immune cell apoptosis (Ayala & Chaudry, 1996). A preponderance of IL-10 occurs in the plasma of human sepsis patients, and is also present in affected organs of mice (van der Poll *et al*, 1997b, 1995). Recently, the use of anti-inflammatory cytokines has been studied *in vitro*, to demonstrate their down-regulatory effects on LPS-induced MTF mRNA transcription, protein expression, and procoagulant activity (PCA), chiefly by IL-10 treatment (also IL-4, IL-13 and TGF-β; Ramani *et al*, 1993a, b; Pradier *et al*, 1993; Jungi *et al*, 1994). IL-10 dose-dependent inhibitions of LPS-induced MTF and PCA in isolated mononuclear cell (MNC) cultures has shown that >5 ng/ml caused a >90% inhibition of MTF or PCA (Ramani *et al* 1993a; Pradier *et al*, 1993; Ernofsson *et al*, 1996), whereas delaying the addition of IL-10 (1 h after LPS) resulted in a reduced inhibition of MTF expression to 68% (Ernofsson *et al*, 1996).

A recent *in vivo* study employing normal human volunteers has confirmed the *in vitro* findings that prior or simultaneous treatment with IL-10 almost abrogates the procoagulant and fibrinolytic responses to LPS challenge (Pajkrt *et al*, 1997). Delayed treatment with IL-10 (1 h after LPS) only partially, but significantly, reduced the procoagulant response, but not the fibrinolytic response (Pajkrt *et al*, 1997). Previous studies employing a mouse model of severe peritonitis showed that delayed delivery of IL-10 (6 h) after sepsis induction decreased lethality (Kato *et al*, 1995).

All these anti-inflammatory treatment regimes aim at intervening in the septic process very early, to inhibit the adverse effects of tissue factor (TF) exposure in peripheral whole blood. The avoidance of the hyperinflammatory phase of sepsis (characterized by an increase in PCA) may also reduce or abrogate the development of the immunoparalysis phase of the disease (Volk *et al*, 1996). The lack of complete inhibition of the LPS-induced procoagulant response when

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IL-10 treatment is delayed may be due to the action of platelets, lymphocytes or granulocytes (Østerud, 1992; Edwards & Rickles, 1980; Levy & Edgington, 1980; Halvorsen *et al*, 1993). IL-10 down-regulates granulocyte and lymphocyte enhancing procoagulant functions, as well as monocyte anti-inflammatory potential (Niiro *et al*, 1997; Snijdewint *et al*, 1993; Faist *et al*, 1996). Specifically, IL-10 up-regulates interleukin-1 receptor antagonist (IL-1ra) expression, which increases monocyte anti-inflammatory effectiveness (Moore *et al*, 1993), while abrogating LPS-induced cyclooxygenase-2 (COX-2) activity and hence granulocyte prostaglandin E_2 (PGE_2) biosynthesis. This causes a reduction of MTF expression and phagocytosis by monocytes and granulocytes (Østerud *et al*, 1990; Niiro *et al*, 1997). Since Th1 helper T-cells are better up-regulators of monocyte PCA than Th2 cells, IL-10 down-regulates MTF expression partly by the switching of the Th1-type cytokine response to Th2 (Del Prete *et al*, 1995; Snijdewint *et al*, 1993; Faist *et al*, 1996).

Platelets, via P-selectin ligation with Sialyl Lewis X, have been shown to induce MTF and activate monocytes (Halvorsen *et al*, 1993; Celi *et al*, 1994; Amirkhosravi *et al*, 1996; Lo *et al*, 1997) which may be resistant to the action of IL-10. Previously, we have shown that in MNC cultures, CD28/B7 signalling up-regulates the expression of MTF in initial phases of the response to LPS, although the delayed addition of CD28-blocking antibodies (1 h after LPS) resulted in a significant reduction in the inhibition of MTF expression (Warnes & Francis, 1996). This may be as a consequence of the lack of B7 ligation with CD28 (constitutively expressed by 95% of helper T cells), as blockade of B7 by CTLA-4Ig is known to inhibit LPS-induced enhancement of monocyte B7 expression, which is down-regulated by IL-10 (Schmittel *et al*, 1995; Ding *et al*, 1993). The other co-stimulatory signalling pathway, CD40–CD154, has also been shown in mixed MNC reactions to induce the expression of MTF, which is resistant to IL-10 (Pradier *et al*, 1996). However, the lack of abrogation of MTF expression by IL-10 (delivered post LPS) may be due to residual low-level monocyte B7 expression.

The aim of this study was to show that IL-10 down-regulates MTF expression in an *in vitro* whole blood model, and that platelet and lymphocyte interactions with monocytes (via P-selectin/Sialyl Lewis X and CD28/B7 signalling) reduces the efficacy of IL-10 in the initial phases of the response to LPS. This may lend support to a modification of the therapeutic use of IL-10 in the treatment of DIC in sepsis.

MATERIALS AND METHODS

LPS-induced dose-dependent response of monocyte TF, B7.1, B7.2, CD40 and helper T-cell CD154 expression in whole blood. Whole blood from normal volunteers was collected on separate occasions into Vacutainers containing 3·2% sodium citrate (Becton Dickinson Vacutainer Systems USA, Rutherford, N.J.). Whole blood aliquots (final volume 1 ml) were incubated on a horizontal shaker for 6 h at 37°C in 5% CO_2 in 24-flat-well non-adherent tissue culture (TC) plates (Costar, Kennebunk, Maine), with or without 0·01, 0·1, 1 or 10 μg/ml LPS (0111:B4; Sigma Chemical Co., St Louis, Mo.). Monocyte TF, B7.1, B7.2, CD40 and helper T-cell CD154 expression was quantified for each LPS concentration by flow cytometry (see section below).

Regulatory role of monocyte B7 and P-selectin on LPS-induced MTF expression and monocyte/platelet binding in whole blood. Citrated whole blood (1 ml final volume) was incubated with 0·1 μg/ml LPS on a horizontal shaker for 6 h at 37°C in 5% CO_2 in the presence of 5 μg/ml recombinant human CTLA-4/Fc chimaera fusion protein (rhCTLA-4Ig; R & D Systems, Minneapolis, Min.), and/or anti-P-Selectin (Endogen, Woburn, Mass.), or IgG_1 murine isotype (R & D Systems, Minneapolis, Min.). Monocyte TF expression and platelet/monocyte binding was quantified by flow cytometry (see section below).

Dose-dependent effects of IL-10 on LPS-induced monocyte TF, B7.1, B7.2, CD40 and helper T-cell CD154 expression in whole blood. Citrated whole blood (1 ml final volume) was incubated with 0·1 μg/ml LPS on a horizontal shaker for 6 h at 37°C in 5% CO_2 in the presence of 0, 0·1, 0·5, 1, 5 or 10 ng/ml recombinant human IL-10 (rhIL-10; R & D Systems, Minneapolis, Min.). For each IL-10 concentration monocyte TF, B7.1, B7.2, CD40 and helper T-cell CD154 expression was quantified by flow cytometry (see section below).

IL-10 down-regulation of LPS-induced MTF expression in whole blood is modulated by LPS-activated platelets and helper T cells. Citrated whole blood (1 ml final volume) was incubated simultaneously with 0·1 μg/ml LPS and 1 ng/ml rhIL-10 (and 1 h after LPS stimulation) in the presence of rhCTLA-4Ig and/or anti-P-selectin (5 μg/ml; isotype control murine IgG_1) on a horizontal shaker for 6 h at 37°C in 5% CO_2. Monocyte TF expression and platelet/monocyte binding was quantified by flow cytometry (see section below).

Flow cytometric determination of monocyte TF, B7.1, B7.2, CD40, helper T-cell CD154 expression and platelet/monocyte binding in whole blood. Incubated whole blood was aliquoted (100 μl) into FACS tubes (Curtin Matheson Scientific Inc., Houston, Texas); MTF was quantitated by incubation with saturating amounts of anti-$CD14^{PE}$ (5 μl; Immunotech, Marseilles, France) and anti-TF^{FITC} (1 μg; no. 4508CJ; American Diagnostica, Greenwich, Ct.) or anti-$IgG_1{}^{FITC}$ (10 μl; Dako, Carpinteria, Calif.) for 20 min at RT; platelet/monocyte binding was quantitated by labelling with anti-$CD14^{PE}$ and anti-$GPIb^{FITC}$ (20 μl; Immunotech, Marseilles, France). Monocyte B7.1, B7.2, CD40 and helper T-cell CD154 expression were quantitated by labelling whole blood aliquots with 1 μg anti-B7.1 (Immunotech, Marseilles, France), anti-B7.2 (Serotec, Raleigh, N.C.), anti-CD40 (Immunotech), anti-CD154 (Immunotech) or IgG_1 isotype control (Immunotech) at room temperature (RT) for 20 min. Aliquots of 2 ml Tris–0·83% ammonium chloride lyse (0·01M; Sigma), pH 7·4, were then incubated with labelled whole blood at 37°C for 20 min. Aliquots for quantitation of MTF and platelet/monocyte binding were then washed twice in PBS and resuspended in 250 μl PBS with 0·25% paraformaldehyde (PFA; Sigma). Monocytes labelled with B7.1, B7.2 and CD40 (or isotype) and helper T cells (labelled with CD154 or isotype) were washed once in PBS and then incubated with 0·5 μg goat anti-mouse conjugated with RED-613 (Gibco

BRL, Paisley, Scotland) at RT for 20 min. After a further wash in PBS, the monocyte tubes (plus isotype) were incubated with anti-CD14^{PE}, with CD154-labelled T cells (plus isotype) incubated with 10 μl CD3^{FITC}/CD4$^{PE\text{-}Cy5}$ (Immunotech) at RT for 20 min. After a final PBS wash, samples were resuspended in 250 μl PBS with 0·25% PFA.

Two thousand monocytes or 4000 helper T cells per sample were acquired by gating on CD14^{PE} or CD3^{FITC}/CD4$^{PE\text{-}Cy5}$ positive events on a Coulter EPICS Elite ESP Flow Cytometer (Coulter Corporation, Miami, Fla.) with correct fluorescence compensation for FITC, PE, RED-613 and PE-Cy5 conjugates. The number of Antibody Binding Capacity (ABC) units of tissue factor, B7.1, B7.2, CD40, GPIb or CD154 per monocyte or helper T-cell population was calculated by incubating saturating amounts of TF^{FITC}, GPIb^{FITC}, B7.1, B7.2, CD40 or CD154 mAb with 'Quantum Simply Cellular^{TM}' (Sigma) beads for 1 h at RT. Beads labelled with co-stimulatory molecule antibodies were incubated with goat anti-mouse^{RED613} (0·5 μg) for 30 min at room temperature and washed in PBS. Labelled beads were acquired on the same instrument settings used for the monocyte or helper T-cell analysis. The accompanying software permits calibration of the PMT2 and 4, converting FITC and RED-613 channel number into ABC units of TF, GPIb, B7.1, B7.2, CD40 or CD154 expressed per monocyte or helper T-cell population respectively. Analysis of histogram data was achieved by use of 'Immuno-4' Coulter software, which permits a channel by channel subtraction of the negative control from the test sample. Hence the percentage positive events and fluorescence channel number in each sample was determined. Converting channel number to ABC values and multiplication of these values by percent positive events allows the determination of total ABC (TABC) in each cell population.

Percent inhibitions for each antigen were calculated from the equation below, except in the case of monocyte/platelet binding were test samples were compared to positive LPS control only.

$$\%\ \text{inhibition} = \frac{\text{TABCstim.} - \text{TABCtest}}{\text{TABCstim.} - \text{TABCresting}} \times 100$$

Statistical analysis. Statistica for Windows (StatSoft, Tulsa, Okla.) was used for statistical analysis. Dependent paired Student's *t*-test was used at 95% confidence limits comparing isotype control to blocking antibody values. Statistical significance was assumed when $P<0{\cdot}05$.

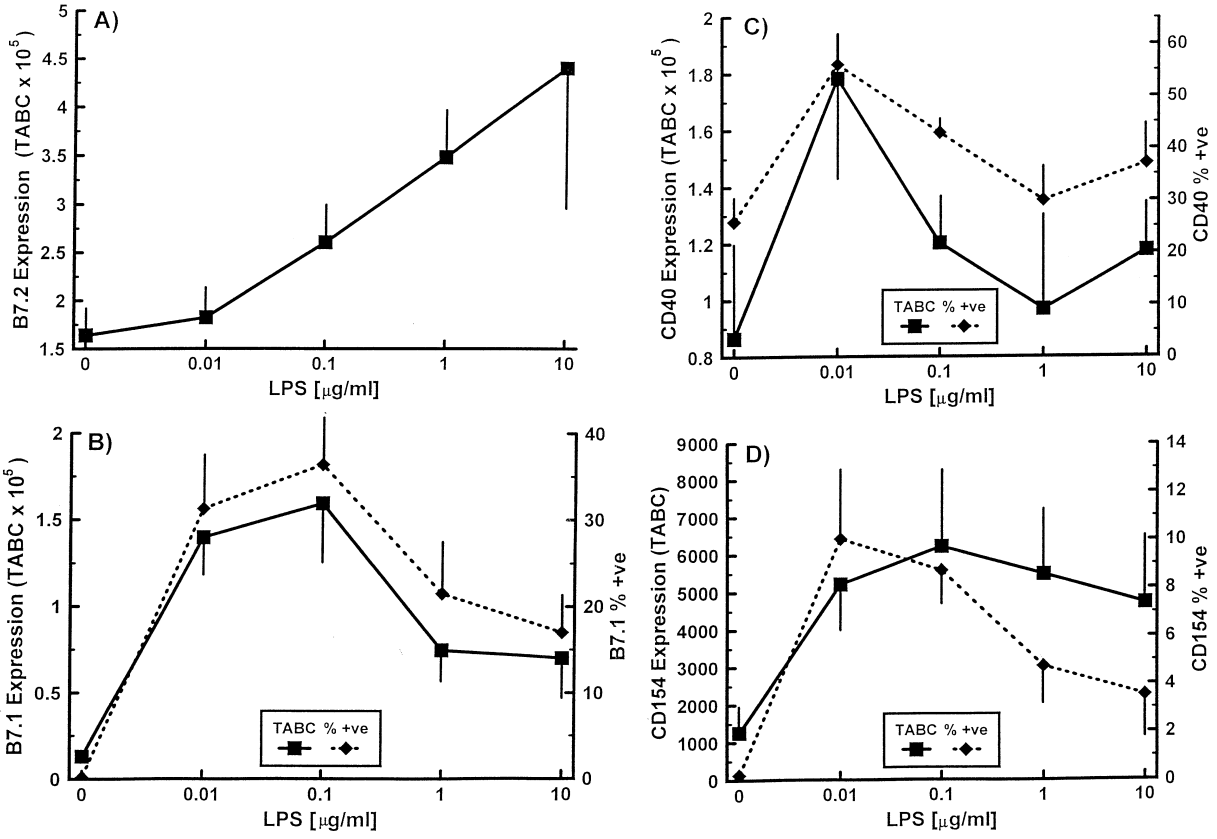

Fig 1. LPS (0–10 μg/ml) dose-dependent up-regulation and induction of: (A) monocyte B7.2 expression (TABC) quantified by 'Quantum Simply Cellular^{TM}' flow cytometric analysis; (B) monocyte B7.1; (C) monocyte CD40; and (D) helper T-cell CD154 expression (TABC) and percent positive events quantified by flow cytometric analysis of CD14^{PE} or CD3^{FITC}/CD4$^{PE/Cy\text{-}5}$ positive events. Whole blood was incubated in ultra-low adherent 24-well TC plates for 6 h at 37°C at 5% CO_2 (n=4). Results are expressed as mean ± SEM.

RESULTS

LPS-induced dose-dependent response of MTF, B7.1, B7.2, CD40 and helper T-cell CD154 expression in whole blood

LPS induced MTF expression in a dose-dependent manner (0·01, 0·1, 1 and 10 μg/ml) in citrated whole blood cultures (Figs 2A and 2B). The induction of MTF (TABC) by LPS ranged from 3·5 to $5·6\times10^5$ (0·01–10 μg/ml); with percentage positive expression of MTF (39–53%) above the baseline control ($1·2\times10^5$ TABC; 11·3%; Figs 1A and 1B).

Constitutively expressed monocyte B7.2 (Azuma *et al*, 1993a) was up-regulated by LPS (resting monocytes 1×10^5 TABC), plateauing at 10 μg/ml LPS ($4·4\times10^5$ TABC; Fig 1A). The other B7 antigen, B7.1 (induced by LPS; Ding *et al*, 1993), was almost zero in the baseline control ($1·3\times10^4$ TABC; 0·2% +ve) and maximized at 0·1 μg/ml LPS ($1·6\times10^5$ TABC; 36·4%; Fig 1B). LPS at higher concentrations (1 and 10 μg/ml) resulted in a lower expression of B7.1 ($0·75\times10^5$ TABC, 21·5% and $0·7\times10^5$ TABC, 17% respectively). Monocyte CD40 up-regulation by LPS peaked at 0·01 μg/ml ($1·8\times10^5$ TABC; 56%) from a baseline ($0·9\times10^8$ TABC; 26%) and falling at the higher concentrations of LPS ($1·2\times10^5$ TABC; 37% for 10 μg/ml LPS; Fig 1C).

The ligand for CD40 expressed by activated helper T cells, CD154 (previously designated CD40L) was present on resting helper T cells in very low amounts ($1·3\times10^5$ TABC, 0·2% +ve, Fig 1D). The percentage of helper T cells expressing CD154 peaked at 10% (0·01 μg/ml LPS), and fell dose-dependently (9–3·5%). The CD154 TABC expression was relatively constant for all LPS concentrations, except at 0·1 μg/ml LPS with maximum expression at 6200 TABC (Fig 1D). The LPS dose-dependent response showed that the high concentration of LPS (0·1 μg/ml) induced relatively high expression of monocyte TF, B7 and helper T-cell CD154 expression, with the exception of CD40 (peaking at 0·01 μg/ml LPS). In the following *in vitro* MTF down-regulation studies the concentration of LPS chosen was 0·1 μg/ml, not only as this gave optimal expression of most co-stimulatory molecules under investigation but allowed comparison to other such studies (Jungi *et al*, 1994; Ernofsson *et al*, 1996; Pradier *et al*, 1996).

Regulatory role of monocyte B7 and Sialyl Lewis X on LPS-induced MTF expression and monocyte/platelet binding in whole blood

We have previously shown that monocyte B7 and Sialyl Lewis X ligation to helper T-cell CD28 and platelet P-selectin

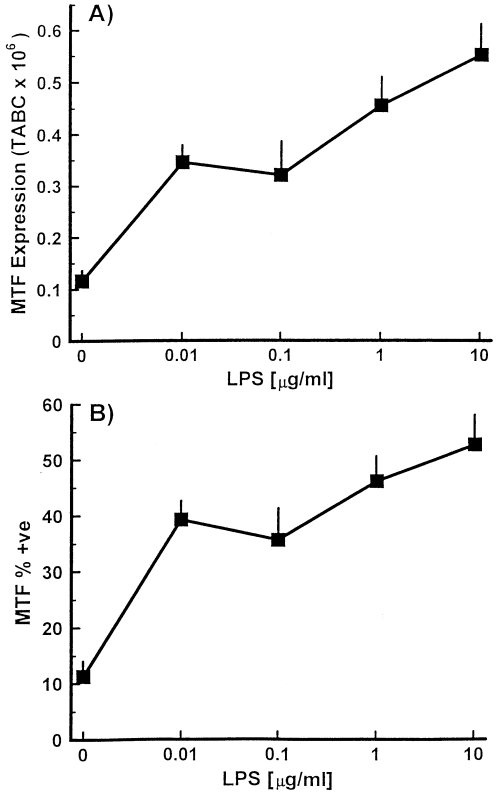

Fig 2. LPS (0–10 μg/ml) dose-dependent induction of MTF in citrated whole blood incubated in ultra-low adherent 24-well TC plates for 6 h at 37°C at 5% CO_2. (A) MTF expression (TABC) quantified by 'Quantum Simply Cellular™' flow cytometric analysis of $CD14^{PE}$ positive events labelled with anti-TF^{FITC}-conjugated antibodies; (B) MTF percent expression (n=5). Results are expressed as mean ± SEM.

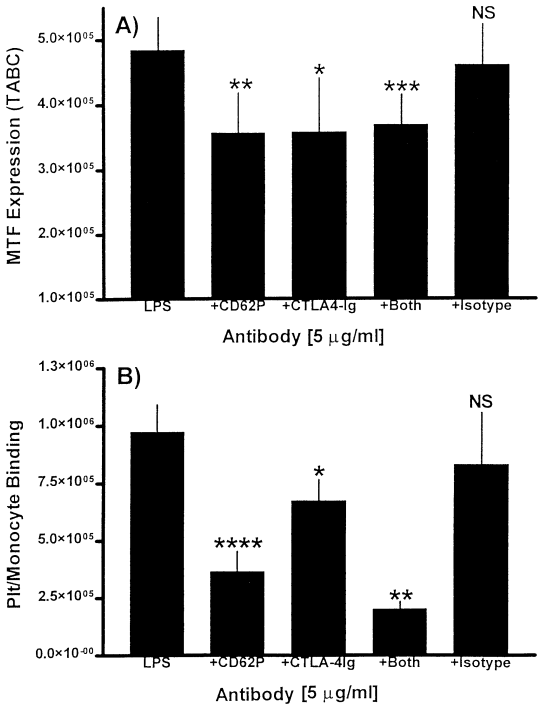

Fig 3. Effect of CTLA-4Ig and/or anti-CD62P (5 μg/ml; n=5; isotype at 5 μg/ml) on LPS (0·1 μg/ml) induced (A) MTF expression; (B) platelet:monocyte binding (Plt/monocyte) in whole blood, by flow cytometric analysis of $CD14^{PE}$ or $CD3^{FITC}/CD4^{PE/Cy\text{-}5}$ positive events was determined by 'Quantum Simply Cellular™' flow cytometry gating on $CD14^{PE}$ events expressing TF^{FITC} or GP^{FITC} (TABC). Whole blood was incubated in ultra-low adherent 24-well TC plates for 6 h at 37°C at 5% CO_2. Results are expressed as mean ± SEM, * P<0·05, ** P<0·01, *** P<0·005, **** P<0·001; NS, not significant (n=5).

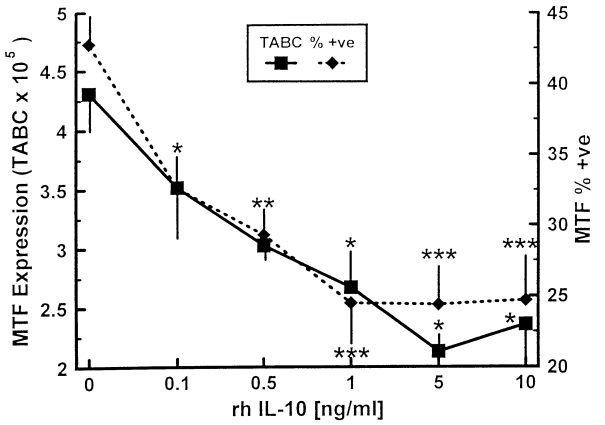

Fig 4. IL-10 (0·1–10 ng/ml) dose-dependent inhibition of LPS-induced (0·1 μg/ml) MTF expression in whole blood determined by 'Quantum Simply Cellular^{TM}' flow cytometry gating on CD14^{PE} events expressing TF^{FITC}. Whole blood was incubated in ultra-low adherent 24-well TC plates for 6 h at 37°C at 5% CO_2 ($n = 5$). Results are expressed as mean ± SEM, * P<0·05, ** P<0·01, *** P<0·005.

up-regulates the MTF response to LPS in a synergistic manner in an isolated cell system (Warnes & Francis, 1996). This was repeated in this study using whole blood incubated with LPS (0·1 μg/ml) in the presence of CTLA-4Ig and/or anti-P-selectin (a reported blocking antibody; Fig 3A). Individually, the blocking agents caused a similar reduction in MTF expression (35%), which was similar to that observed in isolated cell cultures.

However, these blocking agents were not synergistic, contrary to that observed in the isolated cell system, although P-selectin and B7 blockade both affected platelet binding to monocytes (62% and 31% inhibition) with an enhancement of inhibition when both were blockaded (79%; Fig 3B).

Effect of IL-10 dose-dependent response on LPS-induced monocyte TF, B7.1, B7.2, CD40 and helper T-cell CD154 expression in whole blood

The dose-dependent effects of IL-10 on LPS-induced MTF determined in MNC cultures (Ernofsson *et al*, 1996) was confirmed in whole blood (Fig 4). Monocytes expressing TF were reduced in a dose-dependent manner, from 43 ± 3% in the LPS positive control to 24 ± 3% in the presence of 1, 5 and 10 ng/ml IL-10 (P<0·005; Fig 4). This percentage positive MTF expression determined in whole blood with IL-10 compared very well with the reported inhibitions in MNC cultures (Ernofsson *et al*, 1996). The TABC of the monocyte population in such cultures also showed a dose-dependent reduction in MTF expression, with 56%

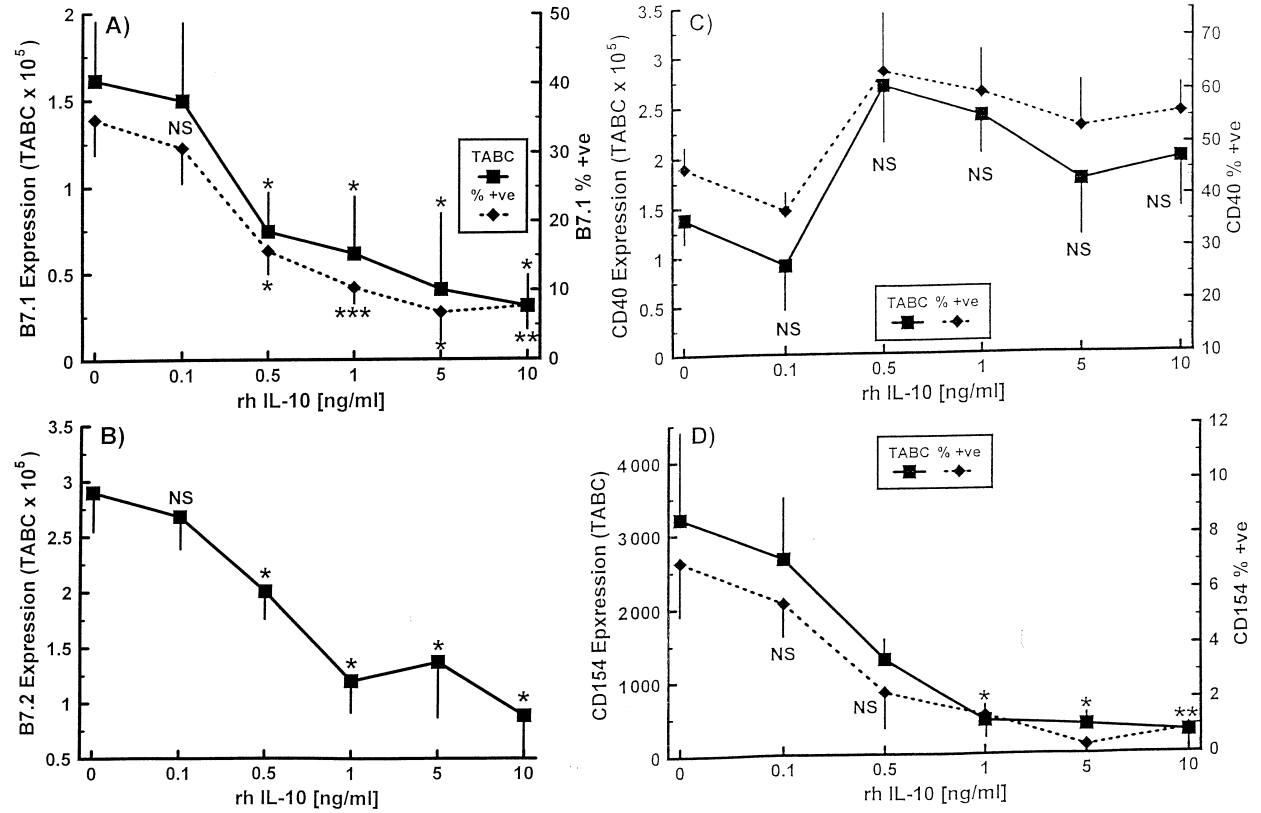

Fig 5. IL-10 (0·1-10 ng/ml) dose-dependent inhibition of LPS-induced (0·1 μg/ml). (A) Monocyte B7.1; (B) monocyte B7.2; (C) monocyte CD40; (D) helper T-cell CD154 TABC expression and percent positive events in whole blood determined by 'Quantum Simply Cellular^{TM}' flow cytometry gating on CD14^{PE} or CD3^{FITC}/CD4$^{PE/Cy-5}$ positive events (n=4). Whole blood was incubated in ultra-low adherent 24-well TC plates for 6 h at 37°C at 5% CO_2 (n=5). Results are expressed as mean ± SEM, * P<0·05, ** P<0·01, *** P<0·005; NS, not significant.

inhibition by 1 ng/ml IL-10, and plateauing to 75% inhibition at the higher concentrations of IL-10 ($2{\cdot}8 \pm 0{\cdot}1 \times 10^5$ TABC and $2{\cdot}1 \pm 0{\cdot}3 \times 10^5$ TABC respectively; $P<0{\cdot}05$; Fig 4). The differences between MNC and whole blood cultures in MTF inhibition by IL-10 may be due to platelet and lymphocyte interactions with LPS-activated monocytes, whereas granulocytes are rendered practically non-functional by IL-10 in terms of their MTF up-regulatory capability (Niiro *et al*, 1997).

To explore this possibility further, the expression of LPS-induced B7.1, B7.2, CD40 by monocytes and helper T-cell activation marker CD154 expression were quantitated in the presence of IL-10 in a dose-dependent manner (Figs 5A–D). B7.1 induction fell to almost zero in the presence of IL-10 (10 ng/ml) ($7{\cdot}7 \pm 3{\cdot}5\%$ positive, $0{\cdot}31 \pm 0{\cdot}16 \times 10^5$ TABC) compared to resting monocytes ($0{\cdot}2\%$ positive and $0{\cdot}1 \times 10^5$ TABC, $<0{\cdot}05$; Fig 5A). Similar reductions in B7.2 expression (below resting levels) was achieved by IL-10 ($0{\cdot}9 \pm 0{\cdot}4 \times 10^5$ TABC) compared to resting monocytes ($1{\cdot}6 \pm 0{\cdot}2 \times 10^5$ TABC; $P<0{\cdot}05$; Fig 5B). The up-regulation of monocyte CD40 by LPS was further enhanced by IL-10, maximally with 0·5 ng/ml (from $1{\cdot}4 \pm 0{\cdot}1 \times 10^5$ TABC to $2{\cdot}7 \pm 0{\cdot}6 \times 10^5$ TABC), whereas higher IL-10 concentrations caused a lowering in monocyte CD40 expression ($P>0{\cdot}05$, Fig 5C).

The LPS induction of helper T-cell CD154 was inhibited in a dose-dependent manner by IL-10, which is known to modulate T-cell Th0/Th1/Th2 responses (Fig 5D). Abrogation of CD154 expression occurred at 5 ng/ml IL-10 ($0{\cdot}3 \pm 0{\cdot}2\%$ positive, 390 ± 176 TABC; $P<0{\cdot}05$; Fig 5D).

Efficacy of IL-10 down-regulation of LPS-induced MTF expression in whole blood is reduced by B7 and Sialyl Lewis X ligation

Previous studies showing the *in vitro* effectiveness of IL-10 to down-regulate MTF expression or PCA has been done in the absence of high numbers of platelets, and the role of lymphocytes excluded by use of adherent monocyte cultures which may have been contaminated with lymphocytes (Ramani *et al*, 1993a; Pradier *et al*, 1993; Ernofsson *et al*, 1996). Thus, the regulatory effects of B7 and Sialyl Lewis X ligation (mediated by T cells and platelets) on LPS-induced MTF expression may explain the reduced effectiveness of IL-10 in whole blood. The 47% reduction in LPS-induced MTF expression by IL-10 (1 ng/ml; Fig 6A) was significantly enhanced by B7 or P-selectin blockade ($P<0{\cdot}05$; Fig 6A). Together, the blocking agents reduced MTF expression to below that produced by the higher concentrations of IL-10

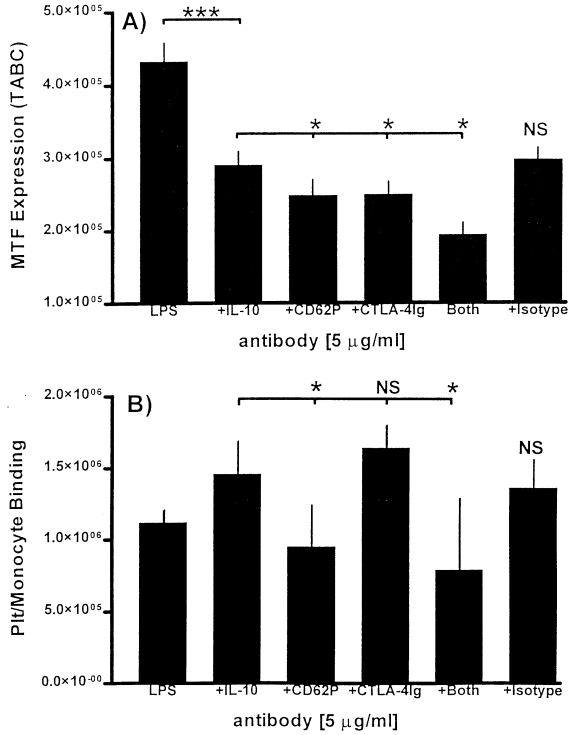

Fig 6. Effect of CTLA-4Ig and/or anti-CD62P (5 μg/ml; $n=5$; isotype at 5 μg/ml) on IL-10 (1 ng/ml) inhibition of LPS-induced (0·1 μg/ml). (A) MTF expression; (B) platelet:monocyte binding in whole blood quantitated by 'Quantum Simply Cellular^{TM}' flow cytometry gating on CD14^{PE} positive events labelled with anti-TF^{FITC} or anti-GPIb^{FITC} ($n=5$). Whole blood was incubated in ultra-low adherent 24-well TC plates for 6 h at 37°C at 5% CO_2 ($n=5$). Results are expressed as mean ± SEM, * $P<0{\cdot}05$, *** $P<0{\cdot}005$; NS, not significant.

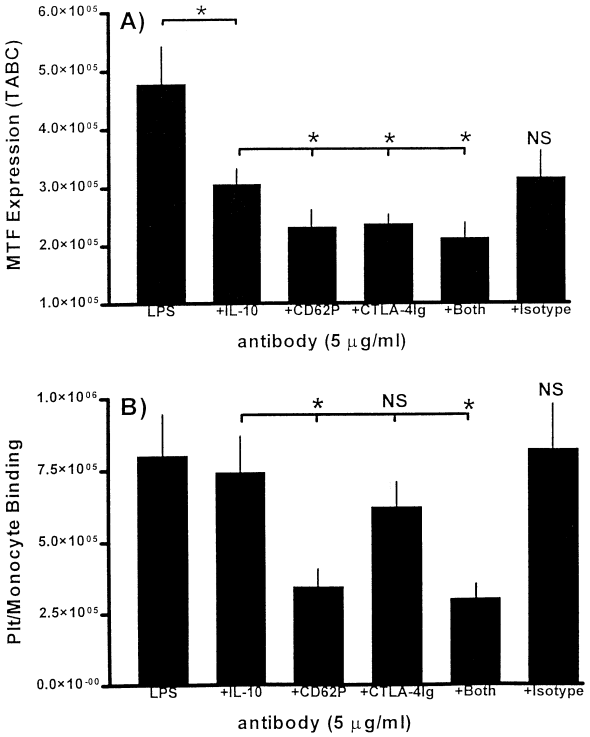

Fig 7. Effect of delayed addition of CTLA-4Ig and/or anti-CD62P (5 μg/ml; $n=5$; isotype at 5 μg/ml) and IL-10 (1 ng/ml) on LPS-induced (0·1 μg/ml). (A) MTF expression; (B) platelet:monocyte binding in whole blood quantitated by 'Quantum Simply Cellular^{TM}' flow cytometry gating on CD14^{PE} positive events labelled with anti-TF^{FITC} or anti-GPIb^{FITC} ($n=4$). Whole blood was incubated in ultra-low adherent 24-well TC plates for 6 h at 37°C at 5% CO_2 ($n=5$). Results are expressed as mean ± SEM, * $P<0{\cdot}05$; NS, not significant.

($1{\cdot}9 \pm 0{\cdot}2 \times 10^5$ TABC compared to $2{\cdot}4 \pm 0{\cdot}3 \times 10^5$ TABC by 10 ng/ml IL-10, $P<0{\cdot}05$; Fig 6A).

The inhibition of platelet/monocyte binding (35%) by P-selectin blockade resulted in a further decrease of MTF expression in the presence of IL-10 ($P<0{\cdot}05$; Fig 6B). This was substantially lower than that determined in the absence of IL-10 (62%; Fig 3B). Blockade of B7 by CTLA-4Ig fusion protein in the presence of IL-10 did not cause a decrease in platelet/monocyte binding, which was contrary to that observed in the absence of IL-10 (Fig 3B). Dual blockade of P-selectin and B7 resulted in a further decrease in platelet/monocyte binding above that observed by P-selectin alone, without effecting expression of MTF (45%; $P<0{\cdot}05$; Fig 6B).

Time-dependent efficacy of IL-10 down-regulation of LPS-induced MTF expression in whole blood

Ernofsson *et al* (1996) employed an isolated cell system and have shown that IL-10 delivered 1 h after LPS caused MTF expression to be 25% higher than when IL-10 was delivered simultaneously with LPS. When IL-10 (1 ng/ml) was added 1 h after LPS in this whole blood model, little difference occurred in MTF expression and platelet:monocyte binding compared to the simultaneous addition of LPS and IL-10 ($P<0{\cdot}05$; Figs 7A and 7B). In the simultaneous addition experiments, blockade of P-selectin and/or B7 resulted similar further reductions of MTF expression and platelet–monocyte binding ($P<0{\cdot}05$; Figs 7A and 7B).

DISCUSSION

The therapeutic use of anti-inflammatory cytokines in the treatment of sepsis is aimed at a reduction or abrogation of the expression of TF by monocytes to limit the development of DIC in the initial hyperinflammatory response (Volk *et al*, 1996). Recent *in vitro* and *in vivo* studies have shown that prior treatment with the anti-inflammatory cytokine, IL-10, before exposure to LPS almost completely negates an increase in MTF expression and end products of PCA (Ernofsson *et al*, 1996; Pajkrt *et al*, 1997). Although the delayed IL-10 treatment (1 h after LPS) caused a significant reduction of MTF expression and the end products of PCA, there was still a significant increase in PCA (Ernofsson *et al*, 1996; Pajkrt *et al*, 1997).

The aims of this study were threefold: (1) to confirm our previous findings of the role of platelet and lymphocyte up-regulation of LPS-induced MTF expression in an *in vitro* whole blood model; (2) to determine that P-selectin and monocyte B7 blockade enhance the effectiveness of IL-10 after exposure to LPS; and (3) hence propose a modification in the therapeutic use of IL-10 to control DIC in sepsis.

Using our whole blood model, flow cytometric analysis confirmed that LPS up-regulated and induced monocyte B7.2 and B7.1 as well as MTF in a dose-dependent manner (Ding *et al*, 1993). The up-regulation by LPS of CD40 on monocytes peaked at a lower concentration of LPS (0·01 μg/ml) than in the case of B7 (0·1 μg/ml). All co-stimulatory molecules analysed plateaued at the higher LPS concentrations (except MTF). LPS, in this 6 h assay, also induced the expression of the CD40 ligand, CD154, on a small percentage (10%) of helper T cells, indicating the participation of T cells in the initial phase of the response to high concentrations of LPS (0·1 μg/ml). In the IL-10 dose-dependent inhibition of MTF, B7.2, B7.1 and CD154, these high levels of LPS enabled comparison with *in vitro* isolated cell studies, with optimal expression for co-stimulatory molecules, as well as relatively high expression of MTF (Jungi *et al*, 1994; Ernofsson *et al*, 1996; Pradier *et al*, 1996).

The MTF regulatory role of platelets and lymphocytes by P-selectin/Sialyl Lewis X and CD28/B7 ligation was confirmed in whole blood (Halvorsen *et al*, 1993; Celi *et al*, 1994; Amirkhosravi *et al*, 1996; Edwards & Rickles, 1980; Levy & Edgington, 1980; Warnes & Francis, 1996). Dual blockade of these antigens did not synergistically inhibit MTF expression, as in the case of isolated cells, but did cause synergistic inhibition of platelet:monocyte binding. The MTF enhancing role of platelets and lymphocytes (and granulocytes) appears to account for the difference in MTF inhibition by IL-10 (5 ng/ml) in whole blood which, at 75%, was lower than that reported previously in isolated cell systems (>90%; Ernofsson *et al*, 1996). IL-10 abrogates granulocyte MTF up-regulatory functions by inhibiting COX-2 and up-regulation of IL-1ra expression by monocytes (Niiro *et al*, 1997; Moore *et al*, 1993). However, the effects of IL-10 on platelets have not been reported, and thus may still be fully functional (or partially due to lack of granulocyte interactions) in their ability to up-regulate MTF expression in whole blood (Halvorsen *et al*, 1993).

LPS (0·1 μg/ml) up-regulation and induction of monocyte B7.1 and B7.2 was down-regulated by IL-10 in a dose-dependent manner, with 1–10 ng/ml causing >50% inhibition. Likewise, helper T-cell CD154 induction by LPS was virtually completely inhibited by IL-10 (between 1 and 10 ng/ml). Thus any MTF up-regulatory potential by the CD154 signalling route (which is IL-10 resistant in mixed mononuclear cell reactions), is negated by down-regulation of helper T-cell CD154 expression, even though monocyte CD40 was up-regulated (Pradier *et al*, 1996). Furthermore, helper T-cell CD28 expression was unaffected by IL-10 (data not shown; de Waal Malefyt *et al*, 1993). Thus CD28-B7 ligation and signalling, if resistant to IL-10, may up-regulate LPS-induced MTF expression.

Consequently, IL-10 (1 ng/ml) with individual and simultaneous blockade of monocyte B7 and platelet P-selectin was chosen to show any enhancement MTF inhibition. Individual blockade caused a further decrease in MTF expression compared to IL-10, whereas the combined blockade acted synergistically to reduce MTF expression below that caused by the higher concentrations of IL-10 (MTF $1{\cdot}9 \pm 0{\cdot}2 \times 10^5$ TABC compared to $2{\cdot}4 \pm 0{\cdot}3 \times 10^5$ TABC [$P<0{\cdot}05$; Fig 5A]). Thus, residual expression of monocyte B7 and presumably full expression of platelet P-selectin during exposure to IL-10 appeared to be able to up-regulate MTF expression.

Delaying the addition of IL-10 and blocking agents by 1 h resulted in little difference in MTF expression compared to when the cytokine was added simultaneously with LPS. The differences in MTF inhibition observed in the IL-10 dose responses and simultaneous addition experiments (56% compared to 46%), plus higher SEMs in the delayed IL-10

experiments explains the lack of difference observed in MTF expression between simultaneous and delayed addition of IL-10. Furthermore, the delayed addition of IL-10 (1 h after LPS) using MNC cultures resulted in only a 25% increase in MTF expression (Ernofsson *et al*, 1996). Given that MTF expression is markedly lower in whole blood models compared to that of isolated MNC, the decrease observed in MTF expression would be small when delaying the addition of IL-10. However, these experiments show that delayed exposure to IL-10 (where B7 expression would be higher), blockade of B7 and P-selectin was still effective in reducing MTF expression to a similar extent as simultaneous addition of IL-10 or the higher concentrations of IL-10.

The effective therapeutic use of anti-inflammatory cytokines or agents that inhibit the inflammatory response in the development of DIC is limited to the early phase of sepsis, with surviving patients later developing an immunoparalysis of the immune system. Recently, it has been shown that anti-TNFα, TNF receptor, IL-1ra or IL-1 receptor treatment has little effect in the treatment of sepsis (van der Poll *et al*, 1994, 1997a; Fisher *et al*, 1994; Preas *et al*, 1996). In murine models, IL-10 has been shown to be effective in the treatment of sepsis, even with a 6 h delay (Kato *et al*, 1995). The injection of normal humans with LPS, who are then given IL-10 to counteract the increase in PCA, does not take into account the bacteraemia which occurs in sepsis patients and their requirement to remove these bacteria. High levels of IFNγ and IL-12 in the hyperinflammatory stages of the disease are associated with lethal effects (Jansen *et al*, 1996). More recently, IFNγ treatment has resulted in a high survival rate of patients with immunoparalysis, caused by the reactivation of their cell-mediated immunity (8/9 patients recovered; Docke *et al*, 1997). Although IL-10 is autoregulatory (Moore *et al*, 1993), treatment of such patients with anti-inflammatory cytokines would be life-threatening, caused by further suppression of the immune system and abrogation of phagocytosis, which presumably would seriously hinder the patients ability to reduce bacterial load (Ertel *et al*, 1996). The high levels of IL-10 detected in non-surviving sepsis patients may limit the usefulness of anti-inflammatory cytokine therapy in long-term sepsis patients (van der Poll *et al*, 1997b).

Helper T cells activated at the onset of sepsis would, in the later stages, express B7.2 and B7.1 (Azuma *et al*, 1993b). Recent studies have shown that T-cell B7.2 preferentially binds to CTLA-4 which appears to inhibit the T-cell response; with helper T-cell B7.1 binding to both CTLA-4 and CD28 (Greenfield *et al*, 1997). Late therapeutic intervention with CTLA-4Ig and IL-10 could also be life-threatening to the patient, due to enhanced inhibition of the helper T-cell response. Conversely, early treatment of sepsis with IFNγ may also have lethal effects. Obviously, if very early intervention is possible with anti-inflammatory cytokines (plus B7 blocking agents) this may reduce the probability of the patient reaching the immunoparalysed phase of the disease, which may be clinically beneficial. Thus, it appears necessary to ascertain which stage of sepsis the patient has reached in order to determine which type of therapy, anti- or pro-inflammatory treatment, is suitable.

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