Multiple Mechanisms of Reduced Major Histocompatibility Complex Class II Expression in Endotoxin Tolerance*

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The exposition of monocytes toward already minor amounts of endotoxin (LPS)† provokes a massive inflammatory response of these cells. However, after initial LPS response, these cells show a modified reaction toward repeated LPS exposure. Such monocytes produce only minor amounts of proinflammatory cytokines, including TNF-α, IL-1β, and IL-12, as well as NO, whereas the production of IL-1 receptor antagonist and TNF receptor II was not altered or actually increased (1–6). This monocyte state after initial LPS priming is therefore designated as endotoxin tolerance. We previously showed that this altered capacity to respond to LPS was paralleled by a strong and long lasting down-regulation of MHC class II expression on these monocytes occurring after a transient up-regulation of this molecule during LPS priming. The reduced MHC class II expression was associated with diminished monocyte T-cell stimulation capacity (7).

Very similar alterations of monocyte function as shown in experimental endotoxin tolerance are also observed in patients after surgery, polytrauma, and septic shock. These patients frequently develop a temporary immunodeficiency, which in its most severe form is referred to as immunoparalysis and predisposes to often life-threatening infections (8). The importance of this phenomenon may be reflected by the fact that in the United States alone more than 200,000 patients die from sepsis each year (9). Reduced monocyte HLA-DR expression is even regarded as a diagnostic marker of this temporary immunodeficiency (8). Unfortunately, there is actually no established causal therapy as yet. However, the study of the molecular basis of experimental endotoxin tolerance may help to better understand the mechanisms of this clinical situation.

We therefore focused on the mechanisms of reduced MHC class II expression in endotoxin tolerance. Expression of antigenic peptide-bearing MHC class II on antigen-presenting cells results from the coordinated interplay of a number of specific events within the cell (10–12). Besides the availability of MHC class II-forming α- and β-chains, various accessory molecules such as invariant chain CD74, cathepsins, and HLA-DM are essential to ensure MHC class II protein structure, transport, and peptide loading. This study addressed these different regulatory levels in human LPS-primed monocytes.

**EXPERIMENTAL PROCEDURES**

Preparation and Culture of PBMCs—Human PBMCs from different healthy donors were isolated from venous blood and cultured as previously described (7). To induce the LPS-primed state in monocytes, PBMCs were cultured in the presence or absence (control group) of 2 ng/ml LPS from Escherichia coli 0112 B8 (Sigma) for 24 h and, if not indicated otherwise, were then extensively washed and recultured for an additional 24–72 h. For comparison of LPS-primed cells at 48 h with cytokine-exposed cells, control PBMCs precultured for 24 h were

- Boxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine; CIITA, class II transactivator; HPRT, hypoxanthine phosphoribosyltransferase-1; RT-PCR, PCR on reverse transcribed mRNA; CLIP, class II-associated invariant chain peptides; MES, 2-(N-morpholino)ethanesulfonic acid.
washed and recultured for the next 24 h in the presence of recombiant TGF-β1 (R & D Systems), IL-10 (PeproTech, Rocky Hill, NJ), or a combination of TGF-β1 and IL-10, each at 10 ng/ml.

To study the effect of blocked cytokine pro tease activities on monocyte MHC class II expression, freshly prepared PBMCs were cultured for 4 or 6 h either in the presence of 25 μg/ml E64d (Sigma), in the respective ethanol concentration (0.05%; v/v) (ethanol control) or without additives (medium control).

For gene expression analysis, Western blot analysis, and immunoprecipitation, monocytes were isolated from PBMCs by CD14-dependent selection using the MACS™ system (Miltenyi Biotec, Bergisch Gladbach, Germany). As judged by flow cytometric analysis, isolated fractions contained at least 95% (gene expression analysis, Western blot analysis) or 90% (immunoprecipitation) monocyte cells.

Enzymatic Digestion of Cell Surface MHC Molecules—Papain treatment of PBMCs was performed as described elsewhere (13) for 2 h using 5 mg/ml papain. Afterward, cells were washed with phosphate-buffered saline and prepared for flow cytometric analysis of MHC class I and II expression.

Flow Cytometric Analysis—For conventional assessment of monocyte surface antigen expressions and purity, PBMCs were stained with the following mAbs according to the manufacturer’s protocol: fluorescein isothiocyanate-labeled anti-HLA-A,B,C (G46–2.6), anti-HLA-DR-D,P,D,Q (Tu39), anti-CLP (ClrClp), mouse IgG1 (MOPC-21), and mouse IgG2a (G155–18). R-phycocerythrin-labeled anti-HLA-DR (L243), anti-HLA-DP,DM,DO,DR (M480), mouse IgG2a (X39), mouse IgG1 (MOPC-21), and mouse IgG2b (X35) (all from Becton Dickinson, Heidelberg, Germany) and R-phycocerythrin-cyanin-labeled anti-CD14 (RMO52, Coulter Immunometric, Hamburg, Germany).

For assessing the monocyte expression of intracellular versus extra cellular HLA-DR, HLA-DM, and CD74 expression, cells were incubated for 20 min at 4 °C in the absence (for detection of HLA-DR, HLA-DM, and CD74) or presence (for detection of HLA-DR and CD74) of the following unlabeled mAbs: 500 μg/ml anti-HLA-DR (Tu36), 25 μg/ml anti-HLA-DR (L243), or 500 μg/ml anti-CD74 (M-B741) (all from BD Biosciences). After extensive washing, cells were either fixed with paraformaldehyde or fixed and permeabilized as described previously (14). Baseline staining was performed using fluorescein isothiocyanate-labeled anti-CD74 (M-B741) and mouse IgG2a (G155–178). R-phycocerythrin-labeled anti-HLA-DR (Tu36), anti-HLA-DR (L243), anti-HLA-DM (M480), mouse IgG2b (27–35), mouse IgG2a (X39), and mouse IgG1 (MOPC-21) (all from BD Biosciences) as well as with R-phycocerythrin-cyanin-labeled anti-CD14 (RMO52, Coulter Immunometric, Germany).

To determine the level of monocytic cathepsin S protein expression, cells were permeabilized as described above and incubated with 2 μg/ml of nonconjugated anti-cathepsin S polyclonal antibodies (C19, Santa Cruz Biotechnology, Heidelberg, Germany) or with the anti-cathepsin S antibodies blocked with the specific peptide used for generation of the antibodies (Santa Cruz Biotechnology). Secondary staining was performed using fluorescein isothiocyanate-labeled donkey anti-goat IgG (H, L) F(ab’2) fragment (Dianova, Freiberg, Germany).

The flow cytometric analyses were performed by means of a FACSort™ instrument and Cellquest™ software (Becton Dickinson). For each measurement, 30,000 PBMCs were analyzed, and monocytes were gated based on their CD14 expression and scatter properties. Data are given as the difference between the MFI of cells stained with the specific mAb and the MFI of cells stained with the respective isotype control mAb or, in case of cathepsin S staining, the peptide-blocked specific antibodies, respectively.

Gene Expression Analysis—Preparation of total RNA and reverse transcription cDNA were performed as described previously (15). cDNA was analyzed in triplicate assays by TaqMan™ PCR by means of the ABI Prism™ 7700 Sequence Detector System (Perkin-Elmer Life Sciences) using TaqMan™ universal master mix (Applied Biosystems, Weiterstadt, Germany) and the following sense primers, antisense primers, and double-labeled oligonucleotide probes: CTTgG gCTTT gATAg C (300 nM), CACCA CgTTC TgCTg cgTgC (300 nM), GGAA CCCTC CATCT AGCTC (900 nM), and FAM-AtcTG gAGTT gATAg CC ACTCA CC (300 nM), GGGAA CGTCA GACG AG AGA TCT (900 nM). The cycle conditions were as follows: 95 °C for 10 s, 60 °C for 1 s, and 72 °C for 1 s for 40 cycles. The melting curve analysis showed one single peak for each product. Results were analyzed using the SDS® 1.2 software (Applied Biosystems). Results were expressed as the difference between the MFI of cells stained with the other known MHC class II-down-regulating mediators, PBMCs were exposed to TGF-β1 or IL-10 or a combination of both. As demonstrated in Fig. 1B, the monocyte HLA-DR expression 24 h after the LPS priming period was, on average, 20.8 ± 0.93% of that of controls and therefore much more down-regulated than in cells treated for 24 h with IL-10 (61.5 ± 4.81% of controls), TGF-β1 (64.7 ± 4.28% of controls), or both cytokines together (48.3 ± 3.45% of controls). In contrast to HLA-DR expression, the effect of LPS priming on MHC class I
TGF-PBMCs were primed with a low dose of LPS at 0–24 h, and monocyte HLA-DR expression was analyzed before (0 h), during (12 h), and after (24–72 h) the priming period. Data are given from one representative experiment as percentage of control (mean ± S.E.). For HLA-DR assessment, the mAb clone L243 was used.

was only marginal (Fig. 1C). Exposition toward TGF-β1 or IL-10 with or without TGF-β1 had little or no down-regulating effect on monocyte MHC class I expression, respectively. LPS priming did not generally reduce monocyte surface protein expression, since it modestly but reliably up-regulated the monocytic marker CD14 (Fig. 1D).

The MHC class II analysis described above was performed using the antibody clone L243, which was used in most studies for detection of a conformation epitope of the mature HLA-DR peptide complex. We tested the expression of other epitopes using additional mAbs. Tu36 detects in any case the HLA-DR αβ epitopes was assessed by flow cytometry using the mAbs Tu36 and Tu39. MFI values from three independent experiments are indicated as mean ± S.E. B, at 48 h, monocyte intracellular HLA-DR αβ content was assessed. The Tu36 epitope expression was analyzed by flow cytometry in permeabilized versus nonpermeabilized monocytes after cell surface epitope saturation using unlabeled Tu36. MFI values from three independent experiments are indicated as mean ± S.E. C and D, before (0 h), during (12 h), and after (24–72 h) the priming period, monocytes were isolated and analyzed for HLA-DR-specific mRNA expression by real time RT-PCR. C, data from one experiment are given as relative to housekeeping gene expression. D, data from four experiments are given as percentage of control (mean ± S.E.).

To assess the cause of down-regulated MHC class II expression in LPS-primed monocytes, we addressed the different regulatory levels of MHC class II expression in these cells. The first prerequisite of HLA-DR expression on cells is the synthesis of this molecule itself. To assess intracellular MHC class II protein levels, we first tried to remove the cell surface fraction by papain digestion. In contrast to the clear reduction of MHC class I, no reduction of monocyte HLA-DR expression was detected after this treatment (data not shown). We therefore decided to block cell surface HLA-DR staining by preincubating cells with an excess of unlabeled Tu36. Thereafter, cells were permeabilized or not and were stained with a fluorescence-labeled Tu36. Fig. 2B demonstrates the results of flow cytometric analysis. In contrast to high levels of intracellular HLA-DR αβ complexes in control monocytes, LPS priming clearly reduced intracellular HLA-DR staining. Nonpermeabilized cells did not significantly stain with this antibody, demonstrating high efficacy of extracellular epitope blocking. Similar data were obtained with Tu39 (data not shown). We further investigated whether the reduced MHC class II protein expression was associated with reduced MHC class II mRNA expression. As assessed by quantitative real time RT-PCR, down-regulation of monocyte HLA-DRα mRNA expression was observed already during LPS priming (at 12 h), peaking at the end of the LPS priming period (at 24 h less than 10% of controls) and recovering the level of untreated controls at 72 h (Fig. 2, C and D). We conclude that monocyte LPS priming provokes a reduced synthesis of MHC class II.
We further studied the expression of molecules known to participate in the intracellular pathway of MHC class II. Freshly synthesized MHC class IIαβ complexes associate in the endoplasmatic reticulum with CD74, which stabilizes their spatial structure, targets them to the endosomal compartments with the help of two sorting signals, and prevents premature peptide loading (10, 11). Monocytes showed low expression of surface CD74, being further reduced in LPS-primed cells (Fig. 3A). Strong intracellular expression of CD74 was detected in control monocytes. LPS priming led to clear down-regulation of the intracellular CD74 protein level. As shown in Fig. 3B, this reduced expression was also observed at the mRNA level (14.3 ± 1.93% of control monocyte expression), suggesting transcriptional regulation of this molecule. CD74 has been shown to be cleaved in a stepwise fashion by lysosomal endopeptidases removing the N-terminal endosomal retention signal and leaving MHC class II groove-binding parts referred to as CLIP. Final CLIP generation resulting from the cleavage of a 10-kDa CD74 fragment, p10, seems to be most critical, being realized only by specialized cysteine endopeptidases. We addressed the role of these enzymes in the regulation of monocyte MHC class II expression. The exposure of murine and human B-cell lines to the cysteine protease inhibitor leupeptin has been shown to induce p10 accumulation and to reduce the transport of freshly synthesized MHC class II to the cell surface as demonstrated by pulse-chase experiments (17, 18), indicating a critical role of CLIP generation for MHC class II cell surface transport in these cells. We investigated the effect of blocked cysteine protease activity on human monocyte steady state cell surface expression by exposing PBMCs to another cysteine protease inhibitor, E64d, for 4 h. As shown in Fig. 4A, surface MHC class II expression was reduced to about 60%. This reduction was further enhanced by extending the incubation time to 6 h (data not shown). In contrast, no down-regulation of MHC class I was observed in the presence of E64d, indicating a selective role of cysteine proteases in the control of expression of monocyte MHC class I.

The cysteine endopeptidases responsible for the generation of CLIP (cysteine S, L, and F) have been investigated (19–22). In myeloid antigen-presenting cell populations, cathepsin S seems to dominate. Here we show that human blood monocytes express all (cysteine S, L, and F) as assessed by real time RT-PCR (Fig. 4B). Among them, cathepsin S-specific mRNA was about 100- to 1000-fold more abundant compared with cathepsin L and F, respectively. In a kinetic study, we measured the monocyte mRNA and intracellular protein expression of cathepsin S during and after LPS priming by means of real time PCR and flow cytometry, respectively. As shown in Fig. 4, C and D, both mRNA and protein expression were reduced already during and continuously after LPS priming relative to nonprimed controls. 2 days after LPS priming, cathepsin S mRNA expression recovered, whereas protein expression did not. These data may suggest an impaired CLIP generation in LPS-primed monocytes. To investigate whether there is a relative accumulation of CLIP precursor in LPS-primed monocytes, we performed Western blot analysis using anti-CD74 and anti-CLIP antibodies. The anti-CLIP antibodies recognized the CLIP precursors p10 and p22 (Fig. 4E). The specificity of detection was confirmed by the absence of signals when the antibodies were blocked with a 400-fold molar excess of peptide used for the generation of these antibodies (data not shown). In contrast to the strong and moderate reduction of CD74 and p22, respectively, LPS-primed monocytes from all four donors tested showed only hardly reduced amounts of p10. This relative accumulation of CLIP precursor agrees with the reduced activity of CLIP-generating enzymes.

MHC class II loading with antigenic peptides is known to take place in the so-called MHC class II loading compartment with the help of other accessory molecules such as the nonclassical MHC class II product HLA-DM. HLA-DM promotes dissociation of CLIP, stabilization of the empty MHC class II complex, and loading with high affinity peptides (11). We studied the HLA-DM protein expression in control and LPS-primed monocytes. No relevant levels were detected on human monocyte cell surface (Fig. 5A), allowing assessment of intracellular HLA-DM expression levels without blocking of surface epitopes. Monocyte LPS priming induced strong down-regulation of this molecule when compared with controls (Fig. 5A). Reduction of HLA-DM level could also be demonstrated at the mRNA level (10.2 ± 1.76% of controls), suggesting that LPS priming interferes with HLA-DM gene expression in these cells (Fig. 5B). A reduced removal of CLIP from MHC class II αβ complexes in LPS-primed monocytes was further supported by the observation that a higher proportion of cell surface MHC class II was loaded with CLIP in these cells compared with control cells (data not shown).

The final step in the expression of MHC class II is its export to the cell surface (10). To investigate whether alterations of the transport might occur in LPS-primed monocytes, we measured the intracellular and extracellular expression of the L243 epitope and determined the ratios of both expressions in control and LPS-primed monocytes from three donors. As shown in Fig. 6A, LPS priming increased this ratio, suggesting a relative accumulation of intracellular MHC class II compared with that in control monocytes. This accumulation can occur (i) simply because of MHC class II retention due to diminished degradation of retaining CD74 (caused here by reduced expression of cathepsin S) and/or (ii) because of direct alteration of vesicular traffic of mature MHC class II-peptide complexes as previously shown for IL-10 action (23). Because there are studies demonstrating that the L243 antibody might not fail to detect MHC class II when associated with CD74 (23), we investigated whether the L243 was able to detect MHC class II αβ complexes associated with any CD74 fragments in monocytes from our donors used in Fig. 6A. Fig. 6B shows immunoprecipitations with the L243 antibody in control monocytes, LPS-primed monocytes, and blank cell lysing buffer. Apart from HLA-DR, p22 was co-immunoprecipitated. In monocytes from another donor, the p10 fragment could additionally, though hardly, be seen (data not shown). Again, the specificity of CD74 fragment
detection was confirmed by the absence of signals when the antibodies used were preincubated with a 400-fold molar excess of blocking peptide (data not shown). These data suggest that in our monocyte donors the L243 antibody detected not only the mature, peptide-loaded HLA-DR but also the immature, CD74 fragment-loaded HLA-DR. Therefore, the relative intracellular accumulation of HLA-DR αβ complexes in LPS-primed monocytes may be due, at least in part, to associated, retaining CD74 fragments. Further studies must be performed to address the extent of direct alteration of vesicular transport in LPS-primed monocytes.

Finally, since the expression of HLA-DR, CD74 and HLA-DM is controlled by CIITA (24), we investigated monocyte CIITA expression before (at 0 h), during (at 12 h), and after LPS priming (at 24, 48, and 72 h) by means of real time RT-PCR. Fig. 7 shows strongly down-regulated CIITA expression already at 12 h (less than 10% of controls), peaking at 24 h and being recovered at 72 h. Therefore, reduced monocytic expression of HLA-DR, CD74, and HLA-DM observed after LPS priming may be due to reduced CIITA expression in these cells.

**DISCUSSION**

Our data suggest that different steps necessary for cell surface expression of mature MHC class II are impaired in LPS-primed monocytes. Besides the strongly reduced MHC class II synthesis, the expressions of CD74, of the major CLIP-generating cathepsin, and of HLA-DM were altered, and reduced export of MHC class II to the cell surface was observed. The essential role of CD74 in MHC class II surface expression was demonstrated in corresponding knockout mice (25, 26). In these mice, MHC class II is largely retained in the endoplasmic reticulum due either to aggregation or to binding of nascent unfolded protein chains. The importance of cathepsins for MHC class II cell surface expression is not fully understood. On the one hand, cysteine protease inhibitors provoke diminished MHC class II cell surface levels (see Fig. 4A and Refs. 17 and 18), and the increase of cathepsin S activity during DC maturation was proposed to be responsible for the massive increase of MHC class II expression (27, 28). On the other hand, cathepsin S deficiency in murine macrophages and dendritic cells from respective knockout mice show no clear reduction of cell surface MHC class II levels (19, 20). This discrepancy may be explained as follows. (i) Newly generated MHC class II-p10 complexes transiently accumulate upon altered activity of CLIP-generating enzymes, initially resulting in reduced MHC class II surface levels. (ii) These complexes also can reach, although delayed, the cell surface. In case of permanent reduction of cathepsin activity, as in the respective knockout mice, MHC class II surface levels should be normalized even in the absence of compensating enzymes because of its regular arrival and half-life at the cell surface. Inborn HLA-DM-deficient mice show normal levels of cell surface MHC class II that mainly present CLIP (29), although MHC class II molecules loaded with CLIP have been postulated to be less stable than those from four donors by real time RT-PCR. Data are given as relative to housekeeping gene expression. C. PBMCs were primed or not (control) with a low dose of LPS at 0–24 h, and monocytes were analyzed for intracellular cathepsin S expression and expression of cathepsin S-specific mRNA by real time PCR and flow cytometry, respectively, during (at 12 h) and after (at 24–72 h) the LPS priming period. Data from one experiment each are given as percentage of control (mean ± S.E.). D. PBMCs were primed or not (control) as in C. At 24 h, monocytes were isolated and analyzed for cathepsin S-specific mRNA expression by real time RT-PCR. Data from four experiments are given as percentages of control (mean ± S.E.). E. PBMCs were primed or not (control) as in C. At 48 h, monocytes were isolated and analyzed for expression of CD74 and CD74 fragments (N18 epitope) by Western blot analysis. The results from one of four experiments are shown.
loaded with selected antigenic peptides. Coming back to our study, we suppose that even if defects in some isolated steps necessary for MHC class II-peptide complex expression may be compensated, their simultaneous inhibition, as presented here in the presence of LPS, results in strong impairment of peptide presentation.

The effect of LPS priming on monocyte MHC class II expression was even stronger than that of the prominent immunosuppressive cytokines IL-10 and TGF-β (Fig. 1B). In contrast to LPS, IL-10 has been excluded from interfering with synthesis of HLA-DR and CD74 (23). Very recently, Fiebiger et al. (28) also excluded any effect of IL-10 on constitutive cathepsin S activity using in vitro generated human dendritic cells. Therefore, the IL-10-induced reduction of MHC class II cell surface expression in monocytic cells may be due solely to altered cellular transport of mature MHC class II as proposed by the group of de Waal Malefyt (23). The same authors also showed that IL-10 does not affect HLA-DM synthesis and peptide loading of HLA-DR. TGF-β has been demonstrated to down-regulate MHC class II mRNA level (30). To our knowledge, no data exist about the effect of TGF-β on other regulation levels of MHC class II expression on monocytic cells. However, the action of TGF-β via alteration of the class II transactivator, which also plays a certain role in HLA-DM and CD74 gene expression, leads us to suppose that TGF-β may also attenuate the expression of these genes (24, 31). Since IL-10 and TGF-β regulate MHC class II expression at different levels, they should act in an additive manner. However, the fact that the combination of both cytokines may not affect all considered levels of MHC class II expression regulation might explain their weaker down-regulating effect compared with LPS priming (Fig. 1B).

Regarding the clinical situation, the results of this study may help explain an important aspect of the pathomechanism in postinflammatory immunodeficiency. In fact, although additional mechanisms other than endotoxinemia may also contribute to the genesis of immunoparalysis, the functional alterations of monocytes in such patients are very similar to those of in vitro LPS-primed monocytes. Conversely, the understanding of the molecular basis of endotoxin priming-induced impaired MHC class II expression may also be useful for the develop-
ment of novel therapeutic approaches in diseases related to overwhelming MHC class II-dependent immune responses such as autoimmune diseases and transplant rejection. The fact that immunoparalytic patients after breaking off immunosuppressive therapy do not reject transplants demonstrates that such approaches should be in principle possible. For treatment of immunoparalysis, autoimmunity, or transplant rejection, the current goal is the identification of molecular targets that mediate the late, immunosuppressive action of LPS but not its early, proinflammatory effects. However, we are still far from realizing such approaches because of the difficulty in identifying the mechanisms of the late LPS effect as demonstrated recently by Wysocka et al. (32) with respect to the monocyte (secondary) LPS response. Since the alterations in endotoxin tolerance concern different complex monocyte functions such as cytokine production capacity and antigen presentation, it can be assumed that also the underlying mechanisms may be complex. Regarding endotoxin tolerance with respect to reduced MHC class II expression, the mechanisms should be sought outside the proximal LPS signaling elements such as toll-like receptor-4, MD2, and MyD88.

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