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Identification of single nucleotide polymorphisms in hematopoietic cell transplant patients affecting early recognition of, and response to, endotoxin

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Abstract

Hematopoietic cell transplant (HCT) is a life-saving therapy for many malignant and non-malignant bone marrow diseases. Associated morbidities are often due to transplant-related toxicities and infections, exacerbated by regimen-induced immune suppression and systemic incursion of bacterial products. Patients undergoing myeloablative conditioning for HCT become endotoxemic and display blood/plasma changes consistent with lipopolysaccharide (LPS)-induced systemic innate immune activation. Herein, we addressed whether patients scheduled for HCT display differences in recognition/response to LPS *ex vivo* traceable to specific single nucleotide polymorphisms (SNPs). Two SNPs of LPS binding protein (LBP) were associated with changes in plasma LBP levels, with one LBP SNP also associating with differences in efficiency of extraction and transfer of endotoxin to myeloid differentiation factor-2 (MD-2), a step needed for activation of TLR4. None of the examined SNPs of CD14, bactericidal/permeability-increasing protein (BPI), TLR4 or MD-2 were associated with corresponding protein plasma levels or endotoxin delivery to MD-2, but CD14 and BPI SNPs significantly associated with differences in LPS-induced TNF-α release *ex vivo* and infection frequency, respectively. These findings suggest that

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specific LBP, CD14 and BPI SNPs might be contributory assessments in studies where clinical outcome may be affected by host response to endotoxin and bacterial infection.

Keywords

LPS-binding protein (LBP); bactericidal/permeability-increasing protein (BPI); bone marrow transplant; hematopoietic cell transplant (HCT); lipopolysaccharide (LPS); CD14; TNF

Introduction

Hematopoietic cell transplant (HCT) is a life-saving therapy for many malignant and non-malignant bone marrow diseases. HCT regimen-related toxicities and post-transplant infections contribute significantly to post-transplant morbidity and mortality.[1,2] Post-transplant inflammation and infections are likely due to the combined effects of conditioning regimen-induced immune suppression and tissue injury, which permit translocation of bacteria and bacterial products to the systemic circulation.[3,4] Lipopolysaccharide (LPS), often referred to as endotoxin, is an abundant and potently pro-inflammatory constituent of the outer membrane of Gram-negative bacteria, and humans undergoing myeloablative HCT are frequently endotoxemic.[5] LPS leakage across damaged intestinal epithelium may be a significant contributor to the so-called ‘cytokine storm’ after myelo-ablative HCT.[6] Systemic LPS induces production of a range of pro-inflammatory cytokines and chemokines, including TNF-α,[7] CCL2, CXCL10 and CCL5,[8] as well as activation of the inflammasome complex and IL-1 β secretion.[9]

The potential of pM concentrations of plasma endotoxin to contribute to immune pathology in HCT patients reflects the remarkable potency of the immune-stimulating properties of LPS in humans. This potency depends on the ordered actions of several host extracellular and cell surface proteins, including LPS-binding protein (LBP), membrane and soluble forms of CD14, secreted or receptor-associated myeloid differentiation factor-2 (MD-2) and TLR4, which together make possible host detection and response to pM concentrations of endotoxin. In brief, LBP catalyzes extraction and transfer of individual molecules of endotoxin from the outer membrane of Gram-negative bacteria (or from aggregates of purified endotoxin) to CD14, followed by transfer of activating endotoxin to MD-2 and engagement and activation of MD-2/ TLR4 complexes.[10–12] This catalytic role of LBP is most pronounced at relatively low LBP concentrations. When present in higher concentrations, LBP can react stoichiometrically with endotoxin and endotoxin/CD14 complexes to promote clearance of endotoxin without engagement and activation of TLR4.[13,14] While the ability of humans to respond sensitively and robustly to endotoxin is important for timely mobilization of host defense responses against invading Gram-negative bacteria, it also increases the risk of potentially harmful immune responses.

Given the potent bioactivity of LPS, humans express a variety of mechanisms to counteract the effects of endotoxin and endotoxemia; these serve to blunt TLR4 signaling and/or skew cellular responses toward production of anti-inflammatory rather than pro-inflammatory mediators. Such mechanisms include (a) modification of MD-2 and TLR4 surface expression;[15] (b) alteration of relative molar ratios of LBP, CD14 and MD-2;[11,16] (c)

enzymatic detoxification of LPS;[17] and (d) neutralization of endotoxin by cationic antimicrobial proteins and peptides,[18] such as the bactericidal/permeability-increasing protein (BPI), which has high affinity for LPS[12] and is found in high concentrations in human neutrophils. Other factors involved in the recognition of, and response to, bacterial compounds include TLR2 (recognizes bacterial lipoproteins), and TLR adaptor molecules myeloid differentiation primary response gene 88 (MyD88) and MyD88 adapter-like Mal/TIRAP, which enable downstream signaling following TLR engagement. The complexity of host mechanisms mediating and regulating host recognition and response to endotoxin is likely to result in variability between individuals in their response to plasma endotoxin.

One possible source of variability between individuals in levels and/or activity of pertinent LPS recognition and signaling proteins could originate from allelic differences deriving from single nucleotide polymorphisms (SNPs). Among these, certain SNPs within the *BPI, CD14, LBP, Mal/TIRAP, MD-2, TLR2* and *TLR4* genes are associated with genotype-dependent *in vitro, ex vivo* and/or *in vivo* (clinical) phenotypes (see Table 1). It is therefore conceivable that effects of endotoxemia in HCT (or other) patients could be influenced by differences between patients in their genotypes for specific LPS recognition or signaling proteins. To date, there have been no reported studies of the possible association of specific SNP genotypes of plasma-derived endotoxin recognition proteins with the efficiency of recognition and response to plasma LPS.

To address the hypothesis that individual HCT patients differ in their recognition of and response to plasma endotoxin due, at least in part, to SNP-derived genotypic differences in LPS recognition proteins, we designed a new observational cohort study and characterized potentially relevant SNPs of LBP, CD14, MD-2, TLR4, BPI and Mal/TIRAP, and the possible relation of specific SNP genotypes to (a) plasma protein levels; (b) plasma-dependent extraction and transfer of endotoxin to MD-2; and (c) responses to LPS in whole blood.

These studies have revealed specific LBP and CD14 SNPs that associate with alterations in plasma protein levels, plasma-dependent extraction and transfer of activating endotoxin to MD-2 (LBP), and activation of MD-2/TLR4 (CD14). In addition, our studies have revealed a potential association of a BPI SNP with post-transplant infection frequency, suggesting that these SNPs should be monitored in future clinical studies where clinical outcome may be affected by host response to plasma endotoxin.

Materials and methods

Patient characteristics

Cohort I—Patients (n = 48) undergoing myeloablative allogeneic HCT from 2005 to 2009 at Boston Children's Hospital (BCH) or Brigham and Women's Hospital (BWH) were recruited prospectively onto an Institutional Review Board (IRB) approved protocol. All participants and/or legal guardians gave consent or assent as appropriate. Patient and treatment characteristics have been published previously (Supplementary Table S1).[19] Supportive care was per institutional routine.[20,21] Prophylactic oral non-absorbable

antibiotics were administered: bacitracin and polymyxin (BWH) or vancomycin (BCH). Blood counts and cultures were performed in clinical laboratories.

Cohort II—Patients (n = 201) being evaluated for myeloablative or non-myeloablative allogeneic, autologous or syngeneic HCT from 2009 to 2011 at the Dana-Farber Cancer Institute (DFCI), BWH, the University of Iowa and Veterans' Administration Medical Center, Iowa City (UIVAMC) were recruited prospectively onto an IRB-approved study (Supplementary Table S2). Onetime peripheral blood samples were taken before transplant (B—baseline) for SNP genotyping and in vitro assays. DFCI/BWH care guidelines were identical to those of Cohort I. Supportive care was according to institutional routine[20,21] at UIVAMC (prophylactic antibiotics used were levofloxacin 500 mg daily or ciprofloxacin 500 mg q12h). Infection data were derived from Center for International Blood & Marrow Transplant Research day 100 reports. We did not have the statistical power to analyze the effects of SNPs on veno-occlusive disease or diffuse alveolar hemorrhage/interstitial pneumonia owing to low frequencies of these clinical complications in our patient population (Supplementary Table S3).

Blood collection and plasma preparation

Peripheral blood samples were drawn into K2-EDTA or sodium heparin vacutainer tubes (Becton-Dickinson, San Jose, CA, USA). Cohort I samples were collected at baseline (B—before myelo-ablation), d 0 (d 0—day of transplant prior to cell infusion) and d 7, 14, 21 and 28, while Cohort II samples were drawn after enrollment and prior to onset of conditioning. Indicated volumes were centrifuged at 1200 g for 5 min at 4°C, and aliquots of the recovered plasma stored in pyrogen-free tubes (Denville Scientific, Metuchen, NJ, USA) at −20°C.

LPS-induced TNF-α release *ex vivo*

Samples of EDTA blood (Cohort II) were stimulated with 1 ng/ml LPS (List Laboratories, Campbell, CA, USA) in Modified Eagle Medium (Invitrogen, Grand Island, NY, USA) control or medium only, and incubated in pyrogen-free 1.5-ml tubes for 4 h at 37°C, rotating end-over-end, prior to collection of the extracellular medium for subsequent TNF-α ELISA (R&D Systems, Minneapolis, MN, USA or BD BioSciences, San Jose, CA, USA).

ELISAs and measurement of chemokines

ELISAs were used to measure TNF-α, sCD14, sTNF-RI, sTNF-RII (all R&D Systems), BPI and LBP (HyCult, Uden, the Netherlands), according to the manufacturers' instructions. sMD-2 was measured by a customized ELISA as previously described.[22] CCL2, CCL5 and CXCL10 were measured by flow cytometry (MoFlo; DakoCytomation, Glostrup, Denmark) using fluorescent beads covalently linked to corresponding mAbs (Cytometric Bead Array BD Flex Sets; BD BioSciences) and results analyzed with Summit v4.0 software (DakoCytomation).

Quantitative real-time PCR arrays

Peripheral blood (2.5 ml) was collected into PAXgene tubes (PreAnalytiX/Qiagen, Hilden, Germany). Total mRNA was isolated using the PAXgene blood RNA kit and the

RNeasyMinElute cleanup kit, followed by cDNA synthesis using the RT^2 First Strand Kit (all Qiagen) according to the manufacturer's instructions. The equivalent of 1 ng RNA/well was assayed using a customized 84-gene signaling pathway PCR array (Qiagen), according to the manufacturer's instructions. Total RNA was normalized using five housekeeping genes and data analyzed using the $\Delta\Delta C_t$ method.[23] Mean fold changes in gene expression in a given patient were calculated by comparing mRNA levels at baseline (B) with subsequent time-points (d 0 and d 14), with >fourfold increases and decreases classified as meaningful up- and down-regulation, respectively.

SNP genotyping

SNPs were genotyped by allelic discrimination using TaqMan assays following the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Two SNPs were genotyped by direct sequencing: rs6099106 as previously described.[24] PCR reactions were conducted as previously described and sequencing was performed by Functional Biosciences (http://functionalbio.com/web/) Rationale for SNP selection included significant association in previous publications, known missense mutations or haplotype coverage in relevant pathway genes (Table 1). Observed SNP frequencies within Cohort II are shown in Table 2.

Assay of plasma-dependent extraction and transfer of endotoxin to MD-2

Typical incubation mixtures contained 85% (v/v) human plasma/EDTA spiked with 5% (v/v) purified aggregates of metabolically-labeled meningococcal lipooligosaccharide (LOS)[26,27] {[^{3}H]LOS$_{agg}$ (500 cpm; 100 pM (500 pg LOS/ml), final concentration} and 10% (v/v) insect cell (Hi5)-conditioned cell medium with or without His$_6$-sMD-2[27–29] [~5 nM (100 ng bioactive sMD-2 monomer/ml), final concentration] in a total volume of 0.2 ml. Human plasma samples were either from individual donors/patients or from a pool of healthy donors used as a control for each assay. All plasma samples were stored in aliquots at −80°C and used after one freeze/thaw. Plasma + [^{3}H]LOS$_{agg}$±His$_6$-sMD-2 were incubated for 15 min at 37°C followed by dilution 2 × with PBS, addition of 20 μl of a 1:1 slurry of Ni^{2+} FF-Sepharose and incubation on a rotating wheel at 4°C for 60 min. After sedimentation of the Ni^{2+} FF-Sepharose gel by centrifugation at 10,000 *g* for 30 s, the supernatant was removed. The gel was washed once with 0.4 ml ice-cold PBS. The recovered supernatants and resuspended gel (in PBS) were transferred to counting vials to measure the recovered [^{3}H]LOS by liquid scintillation spectroscopy, using a Beckman LS6500 scintillation counter (Beckman Coulter, Indianapolis, IN, USA). Percent capture of [^{3}H]LOS by the Ni^{2+}FF-Sepharose gel was calculated as {cpm recovered in gel/[total cpm recovered (gel + combined supernatants)]} × 100. Percent capture of [^{3}H]LOS in the absence of plasma (incubations contained PBS/1% human albumin instead of plasma) was < 1%. Percent capture of [^{3}H]LOS in the absence of added His$_6$-sMD-2 was 0.5±0.2 (range:<0.1–1.1) and was subtracted from percentage capture observed for the same plasma sample incubated with His$_6$-sMD-2 to calculate His6-sMD-2-dependent co-capture of [^{3}H]LOS by the Ni^{2+} FF-Sepharose gel (i.e. plasma-dependent extraction and transfer of [^{3}H]LOS to His$_6$-MD-2). Data for individual plasma samples are expressed as [% MD-2-dependent co-capture of [^{3}H]LOS with the individual plasma/% MD-dependent co-capture of [^{3}H]LOS with the healthy donor plasma pool] × 100 and, for each individual plasma

sample, represent the mean of *2*–4 independent determinations. MD-2-dependent co-capture of [^{3}H]LOS with the healthy donor plasma pool was measured in each experiment (also testing several individual plasma samples) and was 15.2 ± 2.4 (SD) % (n = 25).

Statistical analyses

Samples with undetectable analytes were assigned a value at half the lower limit of detection.

Cohort I—For most assays, data were analyzed after logarithmic transformation, as this yielded distributions that were more approximately normal. For these data, values were then transformed back to original units and plotted on a logarithmic axis. The Wilcoxon signed rank test for matched pairs was used when comparing values for the same patients at different time points with values compared with baseline (B). The significance of fold-change relative to baseline in gene expression for each gene was evaluated with a paired *t*-test on the log-transformed fold-changes. All P-values are two-sided. mRNA levels were normalized to housekeeping genes and quantified using the ΔΔ comparative threshold (Ct) method,[23] using an analysis tool from Qiagen (http://www.sabiosciences.com/pcr/arrayanalysis.php). Statistical analyses of mRNA expression employed SA Biosciences software (Qiagen) using a Student's *t*-test of the replicate $2^{-\Delta Ct}$ values for each gene compared with baseline (see Supplementary Table S4).

Cohort II—Owing to disproportionate distribution of certain SNP genotypes in our study population, only groups of $n \geq 8$ were considered for statistical comparisons. For two-way comparisons, the two-tailed *t*-test or Mann–Whitney test was applied for parametric or non-parametric distributions, respectively. For three-group analyses, a Kruskal–Wallis test with Dunn's Multiple Comparisons Test was applied (all non-parametric distributions). For both Cohorts I and II, results were considered significant at $P < 0.05$, and indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Statistical significance and graphic output were generated using Qiagen online analysis software (http://www.sabiosciences.com/pcr/arrayanalysis.php), Prism v. 4.0a (GraphPad Software, San Diego, CA, USA) and SAS v. 9.1 (SAS Institute, Cary, NC, USA).

Results

Plasma endotoxin during myelo-ablative HCT is followed by systemic inflammatory responses

We recently reported a high incidence of endotoxemia in HCT patients enrolled in an observational cohort study[19] (Cohort I) with elevated levels of plasma LPS manifest by the completion of myeloablative conditioning (d 0). Changes in monocyte cell surface levels of membrane (m)CD14 (diminished) and TLR4 (increased),[19] and in plasma levels of monocyte chemotactic protein 2 (CCL2; Figure 1a) were also manifest on d 0, consistent with acute host responses to plasma endotoxin during myeloablative conditioning. The regimen-induced monocyte nadir at d 0 and d 7 corresponded with a significant decrease in levels of spontaneous TNF-α production in whole blood *ex vivo* on those days (Figure 1b, c), with both changes normalizing by d 21. Plasma levels of the chemokine CCL5 followed

a similar pattern (Figure 1d), contemporaneous with nadirs and normalization in absolute neutrophil count, platelet counts and BPI, and a spike in IL-6 production.[19] In contrast, plasma levels of LBP rose rapidly, peaking at d 7 and d 14, (Figure 1e), while levels of soluble TNF-α receptors 1 and 2 (sTNRF1/2), sCD14 and the IFN-responsive cytokine CXCL10 also increased. However, these changes were more gradual and sustained (Figure 1f – h). No changes in sMD-2 or TLR2 were observed (data not shown).

Measurement of peripheral blood levels of mRNA derived from a customized panel of 84 inflammatory genes provided further evidence suggesting *in vivo* activation of cellular inflammatory responses by d 0 (Figure 2a; Supplementary Table S4) that generally returned to baseline levels (i.e. mRNA levels prior to initiation of myeloablation) by d 14 (Figure 2b). Species of mRNA that were elevated at d 0 relative to cells collected at baseline and at d 14 included multiple genes involved in the LPS–TLR4 signaling axis (*LBP*, *CD14*, *MD-2*, *TLR4* and *MyD88*), as well as inflamma-some signaling (*CASP1*, *IL1B*) and the acute phase response (*CRP*, *LBP*) (Figure 2c). The in vivo elevation of whole blood mRNA levels for these inflammatory genes is especially remarkable given the marked reduction in blood monocyte levels and *ex vivo* whole blood responses to systemic LPS (e.g. secretion of TNF-α) manifest by d 0 (Figure 1b, c). Taken together, these findings suggest acute *in vivo* endotoxin-induced innate immune activation in patients undergoing myelo-ablative conditioning prior to HCT.

Associations of specific genotypes of LBP with differences in LBP protein plasma levels

Four LBP SNPs were analyzed: two SNPs located within the structural gene of LBP, giving rise to structural alterations in LBP (rs2232613 Pro333Leu; rs2232618 Phe436Leu) and two SNPs located in the promoter region of LBP (rs2232571 and rs2232582; Table 1). Genotype-associated differences in plasma levels of LBP protein were observed for rs2232571 and rs2232613. These differences were statistically significant for the more frequently represented genotypes in the Cohort II patient population (rs2232571: CT>TT; rs2232613: CC>CT) (Figure 3a, c). In contrast, no statistically significant differences in plasma LBP protein levels were detected for the other two LBP SNPs examined (Figure 3b, d).

Differences in plasma-dependent extraction and transfer of endotoxin monomer to MD-2 associate with specific LBP genotypes

Maximal potency of activation of TLR4 by endotoxin depends on sequential interactions of endotoxin with LBP, membrane (m) and/or soluble (s) CD14 and MD-2 (secreted or, more typically, associated with TLR4 on cell plasma membranes). LBP catalyzes extraction and transfer of individual molecules of endo-toxin from aggregated forms of endotoxin to sCD14 or mCD14, which, in turn, allows more efficient transfer of TLR4-activating endotoxin monomers to MD-2 and MD-2/TLR4 complexes.[10–12]

To measure the extraction and transfer of endotoxin to MD-2, we designed a novel assay in which individual plasma samples were spiked with recombinant His_6-MD-2 and aggregates of metabolically labeled, purified [^{3}H] endotoxin of very high and virtually uniform specific radioactivity (see ‘Materials and methods’).[13,25,30] LBP SNP rs2232613 showed genotype-

associated differences in endotoxin extraction and transfer to MD-2 that seemed inversely related to genotype-associated mean plasma LBP levels (compare Figures 3c and 4c). Only the differences in MD-2 transfer between the most frequently represented genotypes of LBP rs2232613 (CT > CC; Figure 4c) were statistically significant. The other three LBP SNPs were not associated with any significant differences in endotoxin extraction and transfer to MD-2 (Figure 4a, b, d).

CD14 genotypes confer differences in cell activation by LPS in whole blood *ex vivo*

Two CD14 SNPs (rs2569190 and rs2569191) were analyzed, the former located within the promoter region of the CD14 gene (Table 1). Neither genotype-associated differences in CD14 protein levels in plasma nor in plasma-dependent extraction and transfer of [^{3}H] endotoxin to His_6-MD-2 were observed (Figure 5a – d). However, comparison of LPS-induced secreted TNF-α levels *in vitro* for CD14 SNPs showed a significant difference in TNF-α production between the AG and AA alleles for CD14 SNP rs2569190 (Figure 5e) and between the CC genotype of CD14 SNP rs2569191 compared with either the CT or the TT genotypes (Figure 5f). No effect on LPS-induced TNF-α production from peripheral blood was observed for any of the other SNPs investigated, including TLR4 SNPs rs4986790 and rs4986791 (data not shown).

BPI SNP rs4358188 genotype is associated with increased frequency of infection following autologous and syngeneic HCT

Infection outcome data for patients in Cohort II were available for all patients that underwent HCT (*n*= 188; Supplementary Table S2 and S3). In order to assess whether the SNPs studied were associated with differences in post-transplant infections, we restricted analysis to those patients receiving autologous and syngeneic transplants and, thus, in contrast to those receiving allogeneic cells, retained their SNP genotypes in the hematopoietic compartment (*n*=54). Of the 54 patients receiving autologous or syngeneic transplants, 21 patients had documented infections, all of whom experienced bacterial infection alone, or bacterial plus either viral or fungal infection (Table 3). Four BPI SNPs were analyzed: two located within the structural gene of BPI, giving rise to structural variants of BPI (rs1341023 Ala16Val; rs4358188 Glu216Lys), one located within the 3'-UTR (rs1131847) and one located within an intron (rs6099106) (Table 1). Consistent with prior studies linking BPI genotype with risks of infection,[31,32] there was a decreasing association of infection rates with BPI SNP rs4358188 genotypes AA, AG and GG (*n*=54; Table 4). The limited numbers of patients with infections precluded a sub-group analysis by type of bacterial infection. No apparent differences in SNP genotype distributions and infection frequency in autologous and syngeneic HCT recipients were observed for the other three BPI SNPs or for CD14, LBP, Mal/TIRAP, MD-2, TLR2 or TLR4 SNPs (data not shown).

Discussion

In this study we observed significant changes in multiple innate immune mediators, including TNF-α, CCL2 and CCL5, as well as components of the LPS-detection machinery at the mRNA (CD14, LBP, MD-2, MyD88, TLR4) and protein (LBP, sCD14) levels in

patients undergoing myeloablative HCT. Based on these and our previous observations for BPI,[19] we studied another prospective cohort of patients undergoing HCT to analyze the potential effects of different SNP genotypes in BPI, CD14, LBP, Mal/TIRAP, MD-2, TLR2 and TLR4 on protein levels, functionality and infection outcomes in this population.

We observed two distinct temporal patterns of change in Cohort I. There were components that increased or decreased at early-to-mid-range time points (d 0–d 14) with rapid recovery and those that increased at later time points (d 14–d 28). Components in the early d 0–d 14 group, which includes CCL2, CCL5 and spontaneously secreted TNF-α, corresponded closely to nadirs and recovery of platelets[19] and monocytes, and signify shifts similar to those we have reported previously for neutrophils and neutrophil constituents.[19] We hypothesize that changes following the second pattern, such as the d 14–d 28 increases in LPS receptor components CD14 and LBP, as well as sTNFR1/2 and CXCL10, which are up-regulated in response to Gram-negative bacteria,[8] are suggestive of inflammatory responses that might be influenced by the pattern of hematopoietic ablation and recovery. These can be further exacerbated by the presence of systemic endotoxin,[19] and potentially by clinically evident post-transplant infections.

It should be noted that levels of endotoxemia observed during this time in the HCT patients in Cohort I ranged from intermediate levels of endotoxin activity units (≥ 0.4 and < 0.6; 4/18, 22%) to high levels (≥ 0.6; n = 12/18, 67%),[19] as classified for clinical relevance in the 2003 Multi-Center Endotoxin Detection in Critical illness (MEDIC) trial, where endotoxin units ≥ 0.6 conferred a significantly higher risk of developing severe sepsis within 24 h of testing in Intensive Care Unit patients.[33] At the mid-to-low range of concentrations, the immune-stimulatory activity of plasma endotoxin is likely more dependent on the levels and activity of several host proteins that regulate delivery of LPS to MD-2/TLR4 and/or cell signaling following TLR4 activation. By monitoring several SNPs within genes encoding these host proteins in a second observational cohort (Cohort II), we have found certain SNP genotypes of LBP and CD14 that modify the plasma concentrationsof these proteins (LBP SNPs rs2232613 and rs2232571) and/or the handling (LBP rs2232613) and response to plasma endotoxin *ex vivo* (CD14 SNPs rs2569190 and rs2569191).

Our findings that LBP SNPs rs2232571 and rs2232613 were associated with changes in plasma LBP protein concentrations confirm two previous reports linking rs2232613 and rs2232571 to LBP serum[34] and plasma[35] levels, respectively. Detailed mapping and mutational studies of the *LBP* promoter region have suggested an effect of the C/T substitution in SNP rs2232571 on the function of the CAAT box at position —778 and thus on LBP promoter activity.[36] By showing effects on mean plasma LBP concentrations, our findings (Figure 3a) and those of an earlier study[35] support the hypothesis that LBP SNP rs2232571 affects the *LBP* promoter, with SNP genotype frequency ranking TT > CT > CC inversely relating to protein levels (CC > CT > TT). It is interesting to note that although a common four-SNP risk haplotype in the 5'-flanking region of the LBP gene (positions –1978 to –763, rs2232571 is at position –836) has been linked to an increased risk for severe sepsis,[37] we did not find any association of this SNP with post-transplant infection frequency (albeit in a small cohort). How SNP rs2231613 confers alterations in circulating LBP protein levels (Figure 3c) is less clear, but it is conceivable that the Pro333Leu

substitution leads to structural alterations affecting protein secretion, stability and/or plasma half-life. Whatever the basis of SNP rs2232613 effects on protein levels, this structural alteration appears to be associated with different handling of plasma endotoxin, as manifested *ex vivo* by genotype-associated differences in extraction and efficiency of TLR4-activating endotoxin monomer to MD-2 (Figure 4c). The apparently inverse relation within this LBP SNP of genotype-associated differences in mean plasma LBP levels and plasma-dependent endotoxin delivery to MD-2 is consistent with *in vitro* and *in vivo* data showing that elevated LBP concentrations blunt—rather than promote—delivery of activating endotoxin to MD-2/ TLR4 and endotoxin-induced inflammation.[16]

The elevated plasma LBP levels observed in all HCT patients by d 7–d 14 (Figure 1e) may also contribute to blunting of blood cell responses to endotoxemia that can persist for at least 28 d. It should be noted, however, that our data do not reveal an overall correlation between plasma LBP protein levels and plasma-dependent formation of endotoxin/MD-2 complexes (Spearman correlation, $P = 0.5$), suggesting a possibly added role of the structural alteration of LBP conferred by the rs2232613 SNP and/or other variables in plasma composition. As no genotype-associated differences in plasma-dependent endotoxin/MD-2 formation were observed within the examined SNPs of CD14, MD-2 and BPI, these three endotoxin-binding proteins—as plasma constituents—do not appear to contribute to differences at baseline in handling of plasma endotoxin by patients scheduled for HCT.

Others have shown both CD14 SNPs rs2569190 and rs2569191 to be associated with alterations in CD14 protein levels in blood[38] and plasma[39] without showing associations with altered LPS-induced TNF-α production *ex vivo*.[40] Inversely, we did not observe any associations of CD14 SNPs rs2569190 and rs2569191 with sCD14 protein levels in plasma, but *ex vivo* stimulations of peripheral blood with LPS revealed significant differences in secreted TNF levels when comparing the AA and AG genotypes for rs2569190 and the CC genotype compared with either CT or TT for rs2569191. These apparent contradictions need to be considered in the context of differences in population demographics, age and disease context: Munthe-Kaas et al.[39] showed that rs2569190 and rs2569191 were associated with serum sCD14 protein levels in Norwegian children up to 2 yrs of age, but not in those older than 2 yrs of age. Others have shown that increased CD14 promoter activity *in vitro* with rs2569190 and rs2569191 did not translate into increased mRNA levels in population of healthy Caucasian blood donors,[41] and that rs2569190 association with increased sCD14 levels in blood was observed before, but not after, LPS exposure in healthy volunteers in Belgium.[38]

Nearly 15% of our study subjects showed heterozygous expression of TLR4 SNP variants Asp299Gly and Thr339Ile derived from the SNP variants A/G and C/T, respectively, but there were no significant differences in cellular response to LPS associated with expression of these TLR4 variants in our whole blood assays. This is in agreement with *ex vivo* human whole-blood stimulation assays[42] and consistent with recent evidence indicating that the greatest functional effects of expression of these TLR4 variants are manifest in settings containing relatively low levels of plasma proteins and in cells expressing cell surface TLR4 without MD-2.[27,43] Taken together, we speculate that the differences in cellular response to LPS in whole blood that we see associated with specific CD14 genotypes could reflect

differences in mCD14 (rather than soluble CD14), thereby affecting the efficiency of delivery of activating endotoxin to MD-2/ TLR4 and/or cell signaling following TLR4 activation.

Among the BPI SNPs examined, we saw no genotype-associated differences in either plasma protein levels or *ex vivo* handling of plasma endotoxin. The examined SNPs include a SNP located within the 3′ UTR of the *BPI* gene previously reported to show genotype-associated differences in plasma BPI levels in a study of diabetics.[44] Notably, the plasma BPI levels we measured were about five-fold lower than those reported in the aforementioned study, raising the possibility of additional ethnic and disease-specific or other pathophysiological variables that could affect plasma BPI levels. Given the very low plasma BPI concentrations we measured in HCT patients, it is not surprising that little or no effect of genetic variation in BPI was observed in either of the two *ex vivo* assays performed. In line with previous observations[31,32] and within the limits of a relatively small number of autologous or syngeneic HCT patients (n=54) with documented infection (n = 21), we observed a potential association of one BPI variant (AA genotype within rs4358188) with overall infection frequency among HCT patients post-transplant that should be further validated independently in a larger cohort. If validated, such an effect could conceivably reflect extravascular roles of BPI following expression on mucosa/epithelia and/or secretion from recruited and activated neutrophils at sites of microbial infection.[45,46]

In summary, our findings suggest that recognition and response to plasma endotoxin may be modified by SNP-derived variables in the levels and/or activity of plasma LBP and cell surface CD14 manifest at baseline in patients scheduled for HCT. Additional and more detailed studies will be needed to test whether the associations we have observed reflect effects of the specific LBP and CD14 SNPs on protein expression, secretion, surface expression, half-life or on specific functional properties of these proteins. However, even in the absence of this more detailed mechanistic knowledge, we suggest that future clinical studies where outcome may be affected by host responses to endotoxin, including observational and treatment studies in myelo-ablative HCT,[47] measure the specific LBP, CD14 and BPI SNPs functionally implicated in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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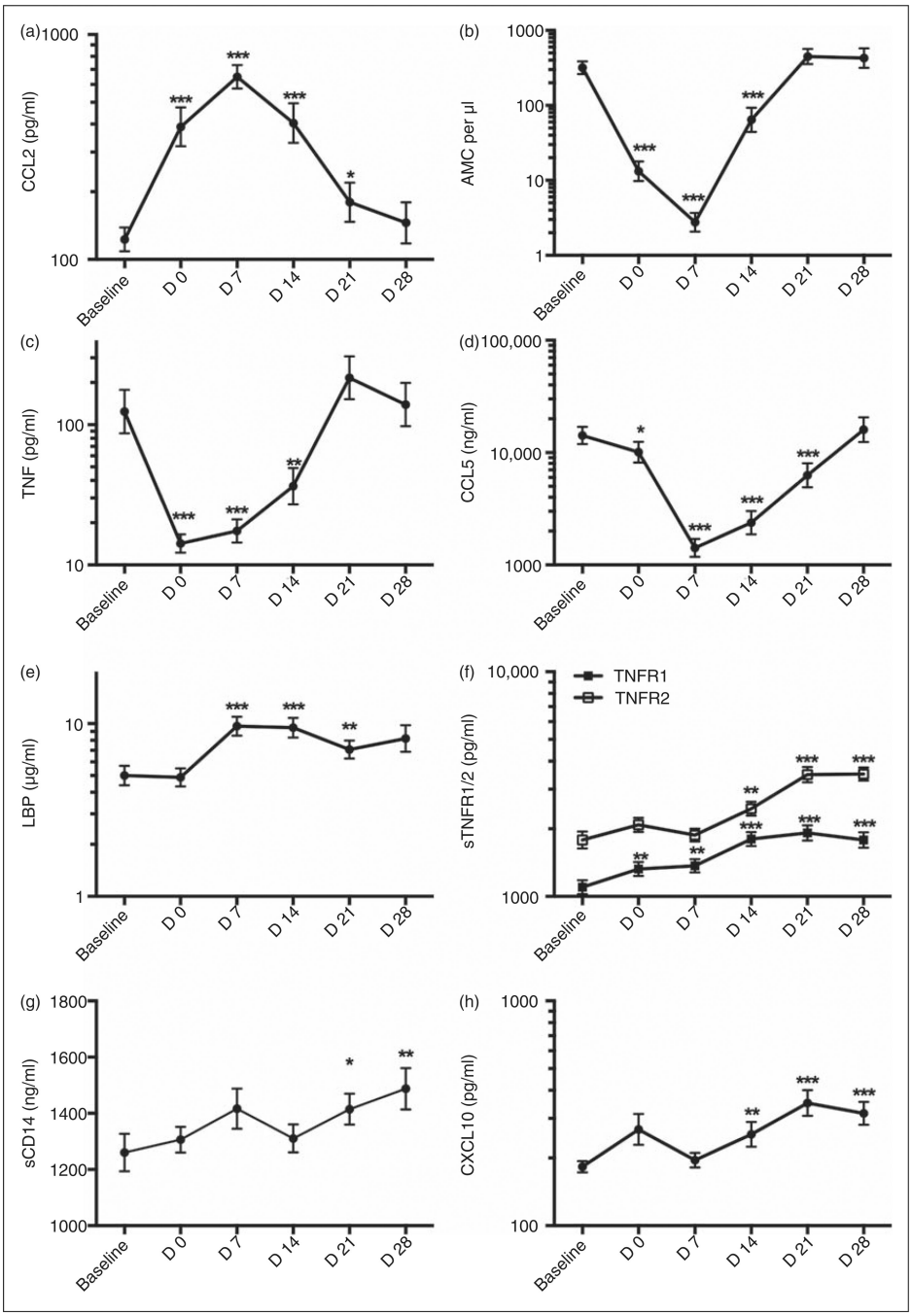

Figure 1.

In vivo and *ex vivo* cellular and plasma changes in inflammatory responses following onset of endotoxemia in myeloablative HCT. Cohort I: peripheral blood and plasma samples from patients undergoing HCT were collected at baseline (B—before myeloablative conditioning), d 0 (day of transplant) and subsequent d 7, 14, 21 and 28, and analyzed for (a) CCL2 protein (pg/ml), (b) absolute monocyte count (AMC) per µl, (c) spontaneously produced TNF-a protein (pg/ml), (d) CCL5 protein (ng/ml), (e) LBP protein (µg/ml), (f) TNF-α receptor 1 and 2 (TNFR1/2) protein (pg/ml), (g) soluble CD14 (sCD14) protein

(ng/ml) and (h) CXCL10 protein (pg/ml) over time. Values are shown as mean ± SEM for n = 22–48 patients. Statistical significance compared to B was assessed using Wilcoxon signed rank test for matched pairs and is indicated as $*P < 0.05$ $**P < 0.01$ and $***P<0.001$.

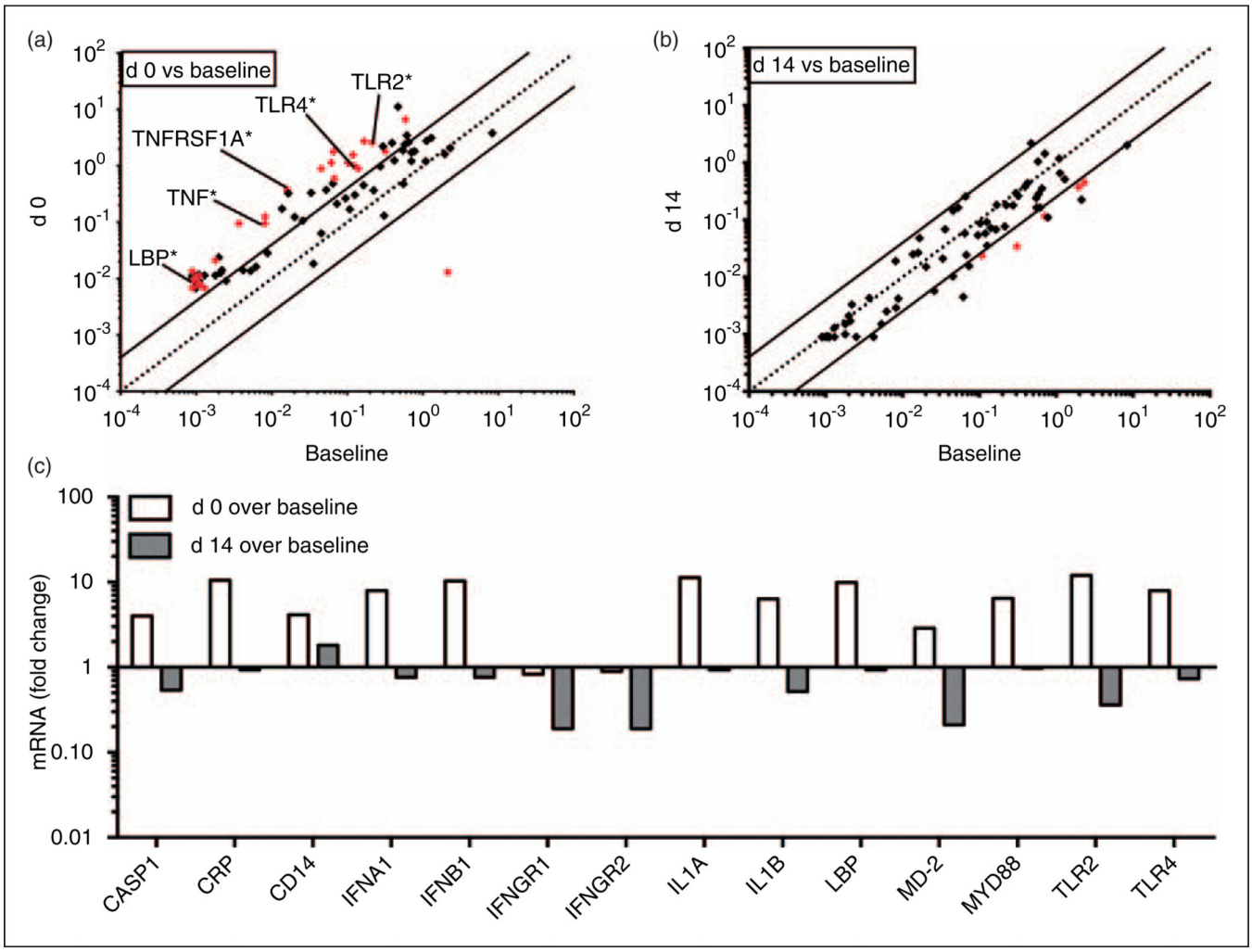

Figure 2.

Changes in mRNA levels of selected inflammatory genes following onset of endotoxemia in myelo-ablative HCT. Cohort I: peripheral blood samples from patients undergoing HCT were collected at baseline (B—before myelo-ablative conditioning), d 0 (day of transplant) and d 14, and mRNA extracted and analyzed for selected inflammatory markers. Fold changes of mRNA levels over B (*x*-axis) on (a) d 0 (*y*-axis) and (b) d 14 (*y*-axis) are shown. (c) Differential regulation over B comparing d 0 and d 14 (from a and b) are shown as bar graphs for a selection of genes. Statistical significance comparing B to either d 0 or d 14 was assessed by Student's *t*-test of the replicate $2^{-\Delta Ct}$ values for each gene compared with baseline. Fold changes greater than fourfold were considered meaningful and statistically significant changes are shown in red for each individual gene (Supplementary Table S4).

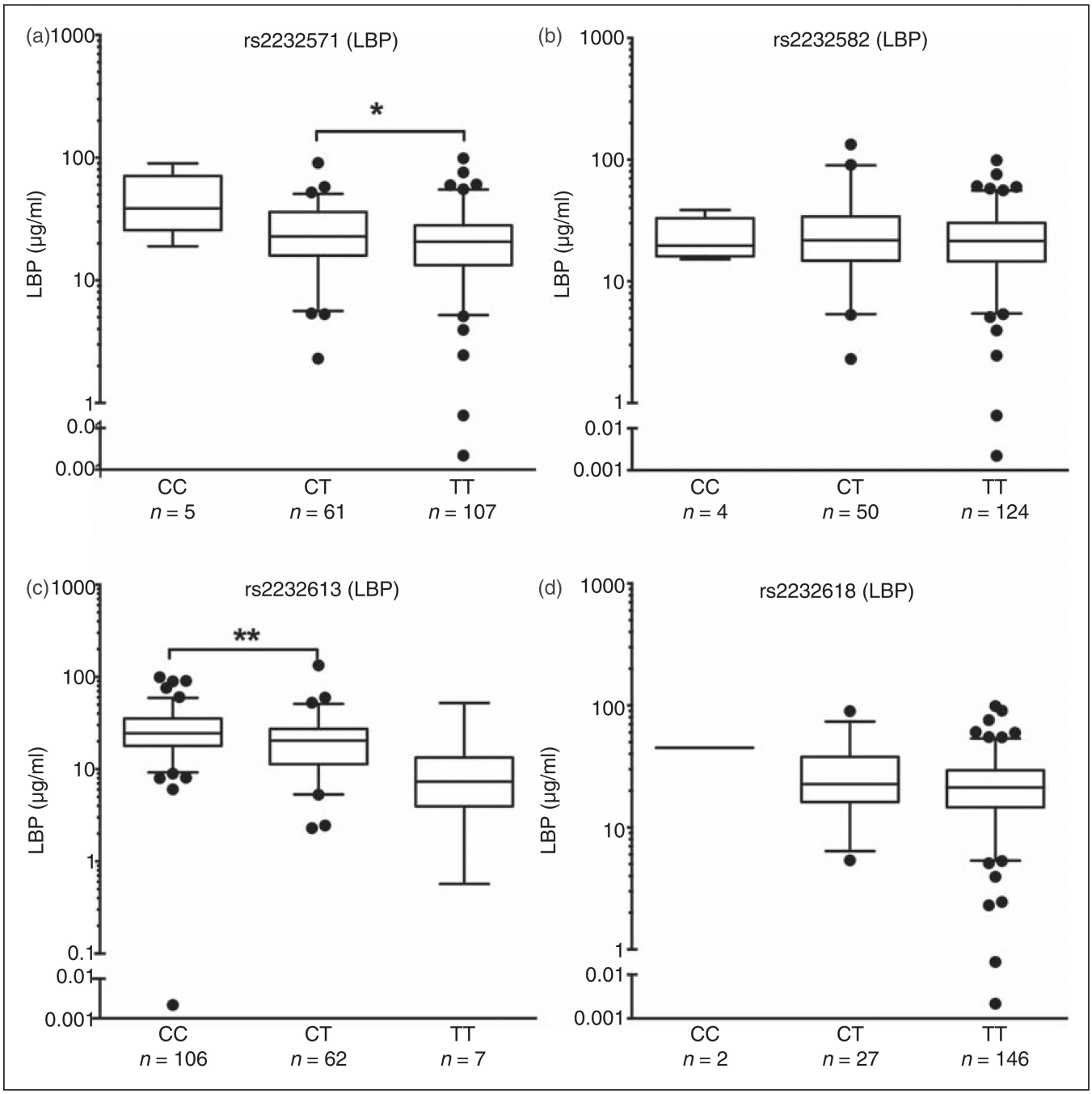

Figure 3.

LBP SNPs rs2232571 and rs2232613 are associated with plasma protein levels. Cohort II: plasma samples corresponding to LBP SNPs (a) rs2232571, (b) rs2232582, (c) rs2232613 and (d) rs2232618 are shown. LBP protein levels in plasma were assessed by ELISA. Values are represented as median and 5–95 percentiles, with individual outliers shown. Only groups of $n \geq 8$ were considered for statistical analyses. Statistical significance was assessed by two-tailed *t*-test or Mann-Whitney test (parametric or non-parametric distributions, respectively) and indicated as $*P<0.05$ and $**P<0.01$.

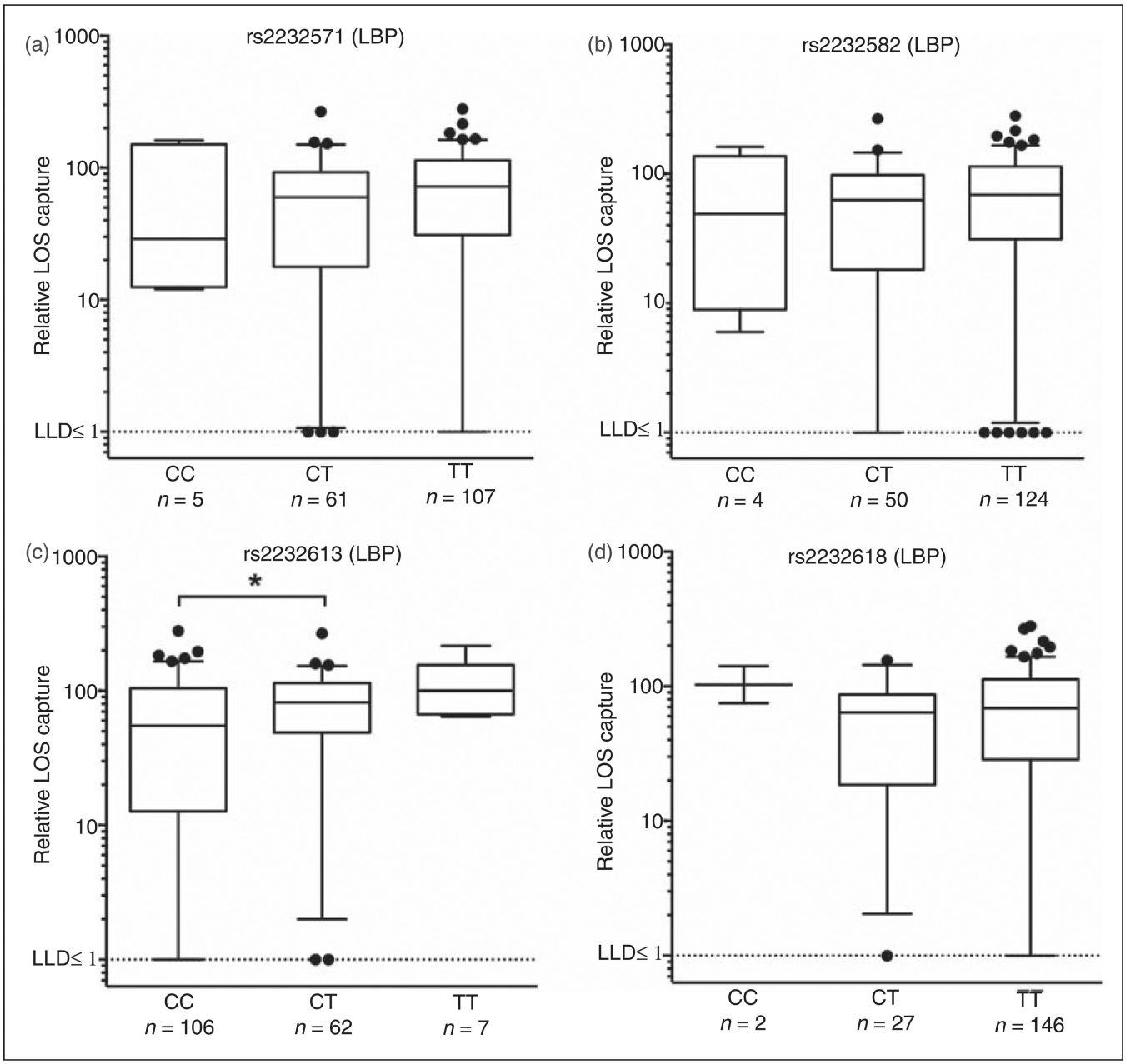

Figure 4.

LBP SNP rs2232613 is associated with altered endotoxin extraction and delivery to MD-2. Cohort II: plasma samples corresponding to LBP SNPs (a) rs2232571, (b) rs2232582, (c) rs2232613 and (d) rs2232618 are shown. Plasma-dependent extraction and transfer of endotoxin monomer to MD-2 was measured and represented as relative LOS capture as described in the 'Materials and methods'. Values are presented as median relative LOS capture (5–95 percentile with outliers shown). Assay detection limit is signified by dotted line (≤ 1%). Only groups of $n \geq 8$ were considered for statistical comparisons. Statistical significance was assessed by two-tailed *t*-test or Mann–Whitney test (parametric or non-

parametric distributions, respectively) and indicated as *$P < 0.05$. LLD: lower limit of detection.

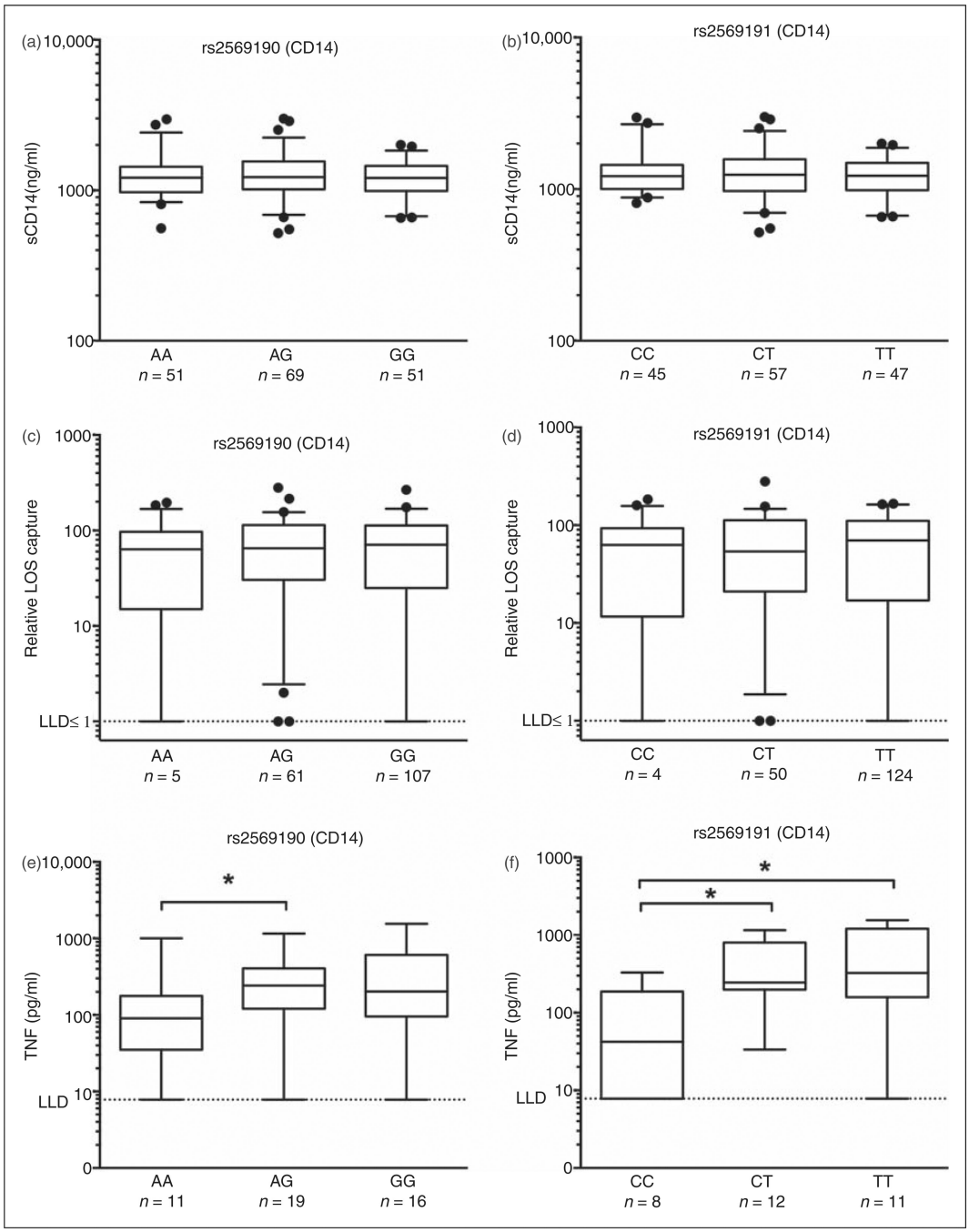

Figure 5.

Association of CD14 SNPs rs2569190 and rs2569191 with altered LPS-induced TNF production *ex vivo*. Cohort II: plasma samples corresponding to CD14 SNPs rs2569190 (a, c, e) and rs2569191 (b, d, f) are shown. sCD14 protein levels were assessed by ELISA (a, b), *ex vivo* plasma-dependent extraction and transfer of endotoxin monomer to MD-2 was measured and represented as relative LOS capture as described in the 'Materials and methods' (assay detection limit signified by dotted line ≤ 1%) (c, d). Extracellular TNF-α accumulation measured by ELISA following *ex vivo* stimulation of whole blood with LPS (1

ng/ml) for 4h (assay detection limit signified by dotted line = 7.8125 pg/ml) (e, f). Values represent median and 5–95 percentile with outliers shown for raw data (a–d) and of log-transformed values labeled in original units (e, f). Only groups of $n \geq 8$ were considered for statistical comparisons. For two-way comparisons (c, d), two-tailed *t*-test or Mann–Whitney test was applied (parametric or non-parametric distributions, respectively). For three-group analyses (a, b, e, f), Kruskal-Wallis test with Dunn's Multiple Comparisons Test was applied. Statistical significance is indicated as $*P < 0.05$. LLD: lower limit of detection.

Table 1

SNPs analyzed in Cohort II.

Gene	SNP	Nucleotides	Phenotype	Findings/clinical correlations
BPI	rs1341023	C/T	Ala16Val	–
	rs4358188	A/G	Glu216Lys	Rapid airflow decline after HSCT (Chien, Zhao et al. 2006)
				LessaGVHD after HCT (Wermke, Maiwald et al. 2010)
	rs1131847	A/G	3′ UTR	Plasma BPI protein levels (Gubern, Lopez-Bermejo et al. 2006)
	rs6099106	C/T	intronic	COPD (Chen, Ou et al. 2012) LPS
CD14	rs2569190	A/G	promoter	Airway inflammation with LPS exposure (Smit, Heederik et al. 2011)
				sCD14 levels in blood (Levan, Michel et al. 2008; Munthe-Kaas, Torjussen et al. 2010)
				Increased promoter activity (LeVan, Bloom et al. 2001; Mertens, Bregadze et al. 2009)
				Survival in ICU patients (Fallavena, Borges et al. 2009)
	rs2569191	C/T	–	sCD14 levels in blood(Munthe-Kaas, Torjussen et al. 2010)
				Airway inflammation (Smit, Heederik et al. 2011)
LBP	rs2232571	C/T	promoter	Severe sepsis (Flores, Perez-Mendez et al. 2009)
	rs2232582	C/T	promoter	Gram-negative bacteremia after HCT (Chien, Boeckh et al. 2008)
	rs2232613	C/T	Pro333Leu	–
	rs2232618	C/T	Phe436Leu	Susceptibility to sepsis and MOD (Zeng, Gu et al. 2012)
Mal/TIRAP	rs8177374	C/T	Ser180Leu	Severe infections after surgery (Kumpf, Giamarellos-Bourboulis et al. 2010)
MD-2	rs6472812	A/G	Gly56Arg	–
TLR2	rs5743708	A/G	Arg753Gln	Reduced responsiveness to TLR ligands (Lorenz, Mira et al. 2000)
	rs1898830	A/G	–	Bronchiolitis obliterans after lung transplantation (Kastelijn, van Moorsel et al. 2010)
	rs1816702	C/T	–	Monocyte activation (Bielinski, Hall et al. 2011)
TLR4	rs4986790	A/G	Asp299Gly	Severe infections after surgery (Kumpf, Giamarellos-Bourboulis et al. 2010)
				Hypo-responsiveness to LPS (Arbour, Lorenz et al. 2000; Guo, Loke et al. 2009)
				Gram-negative bacterial infection (Agnese, Calvano et al. 2002)
				Allograft rejection (Palmer, Burch et al. 2003)
	rs4986791	C/T	Thr399Ile	Severe infections after surgery (Kumpf, Giamarellos-Bourboulis et al. 2010)
				Hypo-responsiveness to LPS (Arbour, Lorenz et al. 2000; Guo, Loke et al. 2009)
				Gram-negative bacterial infection (Agnese, Calvano et al. 2002)
				Allograft rejection (Palmer, Burch et al. 2003)

MOD: multiple organ dysfuction; COPD: chronic obstructive pulmonary disease; ICU: intensive care unit.

Table 2

SNP frequencies in cohort II.

				Frequencies in current cohort		
NCBI number	**Gene**	**SNP**	**Phenotye**	**individuals (%)**	**individuals (n)**	**total (n)**
rs1131847	BPI	A/G	3′ UTR			
		AA		12.50%	25	200
		AG		48.50%	97	
		GG		39.00%	78	
rs1341023	BPI	C/T	Ala16Val			
		CC		22.00%	44	198
		CT		51.00%	100	
		TT		27.00%	54	
rs4358188	BPI	A/G	Glu216Lys			
		AA		26.00%	51	198
		AG		49.00%	98	
		GG		25.00%	49	
rs6099106	BPI	C/T	intronic			
		CC		13.00%	26	200
		CT		53.00%	106	
		TT		34.00%	68	
rs2569190	CD14	A/G	promoter region			
		AA		28.00%	54	192
		AG		41.00%	79	
		GG		31.00%	59	
rs2569191	CD14	C/T	–			
		CC		28.00%	48	170
		CT		40.00%	68	
		TT		32.00%	54	
rs2232571	LBP	C/T	promoter region			
		CC		3.00%	6	194
		CT		35.00%	68	

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				Frequencies in current cohort		
NCBI number	**Gene**	**SNP**	**Phenotye**	**individuals (%)**	**individuals (n)**	**total (n)**
		TT		62.00%	120	
rs2232582	LBP	C/T	promoter region			
		CC		2.00%	4	199
		CT		30.00%	59	
		TT		68.00%	136	
rs2232613	LBP	C/T	Pro333Leu			
		CC		59.00%	116	196
		CT		37.00%	72	
		TT		4.00%	8	
rs2232618	LBP	C/T	Phe436Leu			
		CC		1.00%	2	196
		CT		15.00%	29	
		TT		84.00%	165	
rs8177374	MAL/TIRAP	C/T	Ser180Leu			
		CC		75.50%	149	197
		CT		23.00%	45	
		TT		1.50%	3	
rs6472812	MD-2	A/G	Gly56Arg			
		AA		0.50%	1	194
		AG		8.00%	16	
		GG		91.50%	177	
rs5743708	TLR2	A/G	Arg753Gln			
		AA		4.00%	8	197
		AG		6.00%	11	
		GG		90.00%	178	
rs1898830	TLR2	A/G	–			
		AA		42.00%	81	192
		AG		46.00%	89	
		GG		12.00%	22	
rs1816702	TLR2	C/T	–			

				Frequencies in current cohort		
NCBI number	**Gene**	**SNP**	**Phenotye**	**individuals (%)**	**individuals (n)**	**total (n)**
		CC		76.00%	146	191
		CT		23.50%	44	
		TT		0.50%	1	
rs4986790	TLR4	A/G	Asp299Gly			
		AA		86.00%	172	199
		AG		13.00%	26	
		GG		1.00%	1	
rs4986791	TLR4	C/T	Thr399Ile			
		CC		85.00%	166	196
		CT		14.00%	29	
		TT		1.00%	1	

Table 3

Cohort II infection data.

			All HCT patiens		Allogeneic		Autologous/Syngeneic	
Patients with infections			**(n/total)**	**(%)**	**(n/total)**	**(%)**	**(n/total)**	**(%)**
	Bacteria only		45/188	24%	25/134	19%	20/54	37%
		Bacteria total	77/188	41%	56/134	43%	21/54	39%
	Virus only		9/188	5%	9/134	7%	0/54	0%
		Virus total	40/188	21%	39/134	29%	1/54	2%
	Fungal only		0/188	0%	0/134	0%	0/54	0%
		Fungal total	6/188	3%	5/134	4%	1/54	2%
	Protozoan only		0/188	0%	0/134	0%	0/54	0%
		Protozoan total	2/188	1%	2/134	1%	0/54	0%
	Multiple types of infection		32/188	17%	31/134	23%	1/54	2%
	Total infections		**86/188**	**46%**	**65/134**	**49%**	**21/54**	**39%**

Table 4

Cohort II BPI SNP rs4358188 infection data.

	BPI rs4358188		
	AA	**AG**	**GG**
n/Total	10/15	8/29	3/10
(%)	(66.7%)	(27.6%)	(30%)