Towards precision medicine for septic patients: predicting sepsis-induced hyperinflammation and immunoparalysis.

Internship Report

Bioinformatics

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Abstract

Sepsis is a life-threatening organ dysfunction caused by a dysregulated immune response to infection. It is the number one cause of death in the Intensive Care Unit (ICU), accounting for 6 million casualties every year. Unfortunately, all clinical trials in the last decade failed due to the highly complex and heterogenous pathophysiology of sepsis. Therefore, a shift towards a more individualized treatment approach tailored to the immunological profile of the individual is needed requires a deeper understanding of the genetic mechanisms underlying sepsis heterogeneity and dysregulated immune response. In this study, we integrated genotype, monocyte RNA-Seq and *in vivo* (IV) cytokine data in order to identify transcriptome profiles or expression quantitative trait loci (eQTL) that can be associated with the degree of inflammation upon a first, and endotoxin tolerance upon a second lipopolysaccharide (LPS, 1 ng/kg) challenge in healthy individuals. Transcriptomics analysis revealed 17,094 genes to significantly differentially express between timepoints T=0 and T=4 of which 566 had an absolute log2 fold change > 2. Additionally, inter-individual variability in gene expression was observed leading to the identification of 4,629; 3,739 and 922 genome-wide significant eQTLs at T=0, T=4 and log2 fold change T=0/T=4 (DEG), respectively. The DEG-eQTLs were enriched using the IV cytokine area under the curve (AUC) and 14 expression-cytokine QTLs were identified influencing the expression of 15 genes and in turn IV cytokine response of several cytokines, especially IL-6 and G-CSF. Enriching the eQTLs at T=0 and T=4 using the IV endotoxin tolerance identified two expression-tolerance QTLs for *lnc-LINS-1* and *ECE1*, which may represent genetic predictors for endotoxin tolerance. This study is one of the first studies using an *in vivo* approach to elicit an immune response and quantifying the subsequent degree of endotoxin tolerance acquired. Using the results of this study, we added to the relevance of previous *ex vivo* studies by verifying their findings in human *in vivo*. We gained unique insight into the genetic and transcriptomic fingerprint of cytokine response upon first, and the degree endotoxin tolerance acquired upon second challenge with LPS. These results could be used in the future to make early predictions for a patients chance to develop sepsis-induced immunoparalysis, based on their genetic profile. Further research is required into the involvement of the proposed QTLs as predictors in cytokine response and tolerance acquirement, in order to translate them to clinically viable genetic markers.

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Abbreviations

AUC	Area under the curve
DEG	Log2 fold-change T=0/T=4
EDTA	Ethylenediaminetetraacetic acid
EV	*Ex vivo*
eQTL	Expression quantitative trait locus
ecQTL	Expression-cytokine quantitative trait locus
etQTL	Expression-tolerance quantitative trait locus
G-CSF	Granulocyte Colony Stimulating Factor
GWAS	Genome-wide association study
IL-1RA	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
INT	Rank-based inverse transformation
IP-10	Interferon-γ induced protein-10
IV	*In vivo*
LAF	Least allele frequency
LD	Linkage disequilibrium
LPS	Lipopolysaccharride
MAF	Minor allele frequency
MIP-1a	Macrophage inflammatory protein-1-alpha
PBMC	Peripheral blood mononuclear cell
QTL	Quantitative trait locus
SNP	Single nucleotide polymorphism
TNF	Tumor necrosis factor
cQTL	Cytokine quantitative trait locus
lncRNA	Long-non-coding RNA
tQTL	Tolerance quantitative trait locus

Introduction

Sepsis is a clinical syndrome defined as a life-threatening organ dysfunction caused by a dysregulated host immune response to infection **[1]**. The global incidence of sepsis has been estimated to be higher than 30 million cases per year, resulting in approximately 6 million annual deaths. This makes the disease the number one cause of death in intensive care units worldwide **[2]**. Furthermore, sepsis imposes a significant economic impact on society, ranking among the costliest conditions in hospitals **[3]**.

Unfortunately, all clinical trials including undifferentiated sepsis patient groups performed during the last decades have failed **[4]**. As a result, effective sepsis interventions remain an unmet and urgently required medical need. There is increasing consensus that the difficulty of developing new therapies lies in the highly complex and heterogenous pathophysiology of sepsis. This is hallmarked by both hyperinflammation, and (concurrent) immune suppression, which can lead to a phenomenon called sepsis-induced immunoparalysis **[5, 6]**. Therefore, a shift towards a more individualized treatment approach tailored to the immunological profile of the individual is warranted, requiring deeper insights into the genetic mechanisms underlying sepsis heterogeneity and dysregulated immune response.

In recent years, genome-wide association studies (GWAS) have yielded a vast amount of insight into disease pathophysiology and progression **[7]**. In GWAS, the association between hundreds of thousands of single-nucleotide polymorphisms (SNPs) and complex traits are investigated **[8]**. This has been done, for example, in two GWAS where an attempt was made at identifying associations between SNPs and 28-day sepsis mortality **[9, 10]**. Here, associations with several common polymorphisms were identified, but it remained unclear what these the downstream effects of these SNPs were at the molecular level.

Linking the SNPs with phenotypic data (e.g. an immunological biomarker) allows for the identification of quantitative trait loci (QTLs). A QTL is a region in the DNA that correlates with variation of a particular trait, and it may indicate the genetic basis of phenotypic variation **[11, 12]**. This method can also be applied to variation in genetic expression. By taking genetic expression as a trait, expression QTL (eQTLs) examine the correlation between genetic variation and gene expression levels **[13-15]**. Le et al. took the results of the previously mentioned study and, together with RNA-seq data and sepsis associated SNPs, identified 55

potential genes affected by 39 independent loci **[9, 16]**. Although these studies identified several sepsis associated SNPs as markers for multiple phenotypical observations, they were limited by the extreme heterogeneity and variety among sepsis patients.

Many of these limitations can be negated using the experimental human endotoxemia model **[17]**. In this model, volunteers are challenged with purified endotoxin (as lipopolysaccharide [LPS]) derived from the gram-negative bacterium *Escherichia coli (*E. coli). An intravenous challenge with LPS induces a short-lived and controlled systemic inflammatory response **[18, 19]**. This response exhibits many of the same characteristics as the immune response associated with sepsis **[20]**. When healthy volunteers are first challenged with LPS, an initial pro-inflammatory response is observed, characterized by the release of significant amounts of inflammatory and anti-inflammatory cytokines, along with an increase in blood pressure, heart rate and body temperature, mimicking the initial inflammatory response observed in sepsis. During the second challenge with LPS a week later, the participants exhibit a blunted immune response, known as "endotoxin tolerance". This bears many similarities to sepsis-induced immunoparalysis **[21, 22]**. Harnessing the human experimental endotoxemia model to explore the genetic and transcriptomic landscape of sepsis, enables us to investigate potential biomarkers for diagnosis and treatment for the condition in a controlled and reproducible setup.

In this study, the genotype, monocyte RNA-seq and in vivo (IV) cytokine response data of a cohort of subjects who underwent repeated human endotoxemia were integrated to investigate if there are transcriptome profiles or (e)QTLs that can predict response to the first administration of LPS (analogous to hyperinflammation in sepsis) as well as to the second LPS application and the associated degree of endotoxin tolerance (analogous to sepsis-induced immunoparalysis).

The analysis of the monocyte RNA-Seq revealed 17,094 genes significantly differentially expressing between T=0 and T=4, of these genes, 566 had an absolute log2 fold change > 2. Additionally, inter-individual variability in gene expression was observed leading to the identification of 4,629; 3,739 and 922 genome-wide significant eQTLs at T=0, T=4 and log2 fold change T=0/T=4 (DEG), respectively. Enriching the DEG eQTLs using the IV cytokine response identified 14 expression-cytokine QTLs influencing the expression 15 genes and in turn IV cytokine response of several cytokines, especially IL-6 and G-CSF. The eQTLs at T=0 and T=4 were enriched using IV endotoxin tolerance. Here two expression-tolerance QTLs

were identified for *lnc-LINS-1* and *ECE1*, acting as genetic predictors for endotoxin tolerance. This study is one of the first studies using an *in vivo* approach to elicit an immune response and quantifying the subsequent degree of endotoxin tolerance acquired. Using the results of this study we added to the relevance of previous *ex vivo* studies by identifying them IV. We gained unique insight in the genetic and transcriptomic fingerprint of cytokine response upon first, and endotoxin tolerance upon second challenge with LPS. Using these results, early predictions can be made in order to predict endotoxin tolerance, and in turn sepsis-induced immunoparalysis, based on the genetic profile of sepsis patients. For this to become a reality, further research, for instance functional studies using deletion or overexpression of identified genes, and additional studies into identified SNPs are warranted.

Materials and methods

Study design

The protocol for this study (100LPS study) was approved by the local ethics committee (CMO Arnhem-Nijmegen; reference no. NL68166.091.18 and 2018-4983). One-hundred-and-thirteen healthy volunteers between 18-35 years of age were recruited. All subjects provided written informed consent and were included after medical history, physical examination, routine laboratory tests and a 12-lead electrocardiogram revealed no abnormalities. Smoking, use of any medication (contraceptives precluded), previous participation in experimental human endotoxemia or signs of acute illness within two weeks prior to the start of the study were considered exclusion criteria. All study procedures were performed in compliance with the declaration of Helsinki and its latest revisions.

All subjects were challenged twice with intravenous boluses of 1 ng/kg of LPS one week apart. See Jansen et al., for details of the endotoxemia study procedures **[23]**. During both challenge days, *in vivo* (IV) circulating cytokine responses were determined by frequent blood sampling. The first LPS challenge (day 0) served to quantify the primary cytokine response and to induce endotoxin tolerance. The second LPS challenge (day 7) served to measure the degree of endotoxin tolerance. The area under the plasma cytokine concentration-time curve (AUC) was used as an integral measure of the IV cytokine response over time during each LPS challenge day (Figure 1). The endotoxin tolerance was then quantified by calculating the log2 fold change between the AUC on day 7, and the AUC on day 0. Therefore, more pronounced tolerance is indicated by a more negative log2 fold change value.

Two subjects (029 and 075) were excluded due to being related to another participant. Additionally, eight subjects (007, 011, 023, 032, 038, 040, 044 and 074) were excluded due to being of non-Dutch descent. Subjects 090, 094 and 101 had received a lower dose of LPS and were thus not representative for the cohort and excluded. Lastly, for analyses using endotoxin tolerance, subjects 003 and 012 were excluded for not having measurements on the second LPS challenge day. This leaves 100 participants (49 female, 51 male) for cytokine-, and 98 participants (48 female, 50 male) for tolerance-analysis.

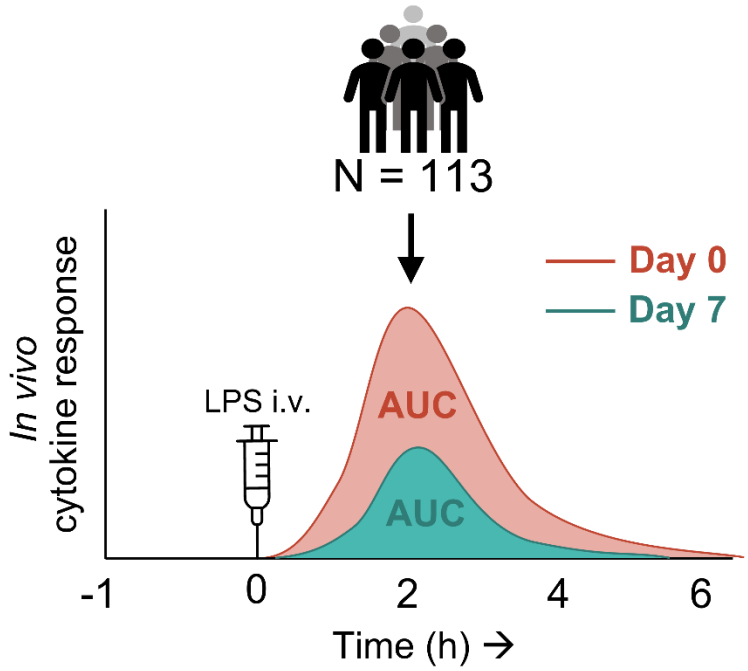

Figure 1. Schematic overview of the AUC, area under the plasma cytokine concentration-time curve.

Cytokine analysis

For plasma cytokine determination, EDTA-anticoagulated blood was centrifuged (10 min, 2000g, 4ºC) directly after withdrawal and plasma was stored at -80ºC until analysis. Concentrations of tumor necrosis factor (TNF), interleukin (IL)-1 receptor antagonist (Ra), IL-6, IL-8, IL-10, macrophage inflammatory protein (MIP)-1α, monocyte chemoattractant protein (MCP)-1, granulocyte-colony stimulating factor (G-CSF) and interferon-γ induced protein (IP)-10 were determined batchwise using a simultaneous Luminex assay (Milliplex, Millipore, Billerica, USA) as per the manufacturer's instructions.

Genotyping the cohort, quality control and imputation

Genomic DNA was isolated from EDTA-anticoagulated blood using a Gentra Puregene Blood Kit. Subsequently, the cohort was genotyped using Illumina Infinium Global Screening v3.0 Arrays. These chips presented the genetic variation in .IDAT format which was first transformed to an intermediary .GTC format using the Illumina Array Analysis Platform Genotyping Command Line Interface (IAAP-CLI) **[24]**. This format was then converted into .VCF format using gtc2vcf **[25]**.

Using Plink 2.0, SNPs with missingness rates of lower than 10%, minor allele frequency (MAF) less than 10% and Hardy-Weinberg equilibrium P < 1^{e}-05 were excluded **[26]**. This left

435,245 variants for phasing and imputation. The variants were prepared for imputation by removing the sex chromosomes and splitting each chromosome into separate sorted vcf.gz files using BcfTools **[27]**. Finally, using the Michigan imputation server, the variants were phased by Eagle v2,4 and imputed using the HRC1.1 EUR population as a reference and Rsq > 0,3 **[28, 29]**. The imputed genotype was again filtered using Plink and the aforementioned parameters. Additionally, a 'least allele frequency' of > 3% was applied in order to remove variants with low coverage for the alternate homogenous allele. This resulted in 3,333,537 and 3,311,421 remaining SNPs for the cytokine and tolerance populations.

Isolation, mapping and pre-processing of classical monocyte RNA

Monocytes were isolated one hour before (T=0) and 4 h after (T=4) both times LPS was administered. To this end, peripheral blood mononuclear cells (PBMCs) were first isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood using Ficoll-Paque (GE Healthcare, Chicago, USA) isolation as described in detail elsewhere **[30]**. Subsequently, CD14+CD16- monocytes were isolated by immunomagnetic negative selection using a monocyte isolation kit (EasySep™ Human Monocyte Isolation Kit, STEMCELL Technologies, Cologne, Germany) as per the manufacturer's instructions. RNA was isolated using RNeasy kits (Qiagen). The isolated samples were sent to BGI (https://www.bgi.com/us/landing/rna-sequencing/) for sequencing using their proprietary DNBSEQ™ platform. Using the RNA-DxP pipeline developed in house by the Center for Molecular and Biomolecular Informatics (CMBI), the resulting paired-end reads (.fastQ) were trimmed using Trim Galore! v0.4.5 to remove low-quality bases and adapters, quality checked using MultiQC and subsequently mapped against the GRCh38 reference transcriptome using STAR v2.6.0a **[31-34]**. The gene counts were quantified using HTSeq v0.11.0, resulting in gene counts for 58,735 genes **[35]**. Counts were filtered on a sum of counts of > 200 over the cohort, leaving 20,843 genes for down-stream analysis. The remaining read counts were then normalized using the trimmed means of M (TMM) values, log2 transformed and rank-based inverse transformed (INT).

Cytokine and tolerance QTL mapping

3,333,357 and 3,311,421 genetic variants were mapped with IV cytokine production and endotoxin tolerance for 100 and 98 participants, respectively. The IV cytokine AUC and tolerance data were rank-based inverse normal transformed and associated with the variants using Matrix eQTL v2.1.0 **[36]**. Gender information was coded either 0 for females or 1 for

males. The gender and use of oral contraception were used as covariables in the association analysis. An explorative approach was applied to the identified cytokine-QTLs (cQTLs) and tolerance-QTLs (tQTLs) and a p-value of $< 5^{e}$-07 was considered to be the threshold for significance.

Genes association with the QTL SNPs were identified using the HaploReg SNP annotation tool v4.2 **[37]**. Here all SNPs in linkage disequilibrium (LD) ($R^2 > 0.8$; using the CEU population as a reference) with the identified c- and tQTLs were extracted. Genes closest to the QTL SNP and genes overlapping with either the QTL SNP or SNPs in LD with the QTL SNP were associated with the QTL. Additionally, associated genes were identified by performing cis-eQTL mapping in a 500-kb window using the RNA-Seq data extracted at T=0, T=4 and the log2 fold change T=0/T=4 (FDR ≤ 0.05).

Exploring the inter-individual differences in the transcriptome

The RNA-Seq data was analyzed and integrated with the genotype in order to gain insight in the transcriptome pre- and post-inflammation and the genetic underpinnings of the inter-individual variability in the transcriptome. Differential expression analysis was done between T=0 and T=4. Using DESeq2 the gene-level expression values were analyzed, and differentially expressed genes identified **[38]**. Absolute log2 fold change > 2 and q-value (Benjamini-Hochberg adjusted p-value) < 0.05 were used as the statistical cutoffs.

GWAS analyses were performed for expression levels at T=0 and T=4. Using Matrix eQTL with gender and use of oral contraception as covariates, expression-QTLs (eQTLs) were identified by associating the gene expression for 20,843 genes with the 3,333,537 variants. To identify inter-individual differential up- or down-regulation of genes between T=0 and T=4 during LPS challenge, the log2 fold change T=4/T=0 (DEG) was also calculated and associated with the variants in a similar manner. For both analyses a genome-wide significance was based on a p-value of 5^{e}-08.

Using Pearson's correlation test, a correlation analysis was performed between the gene expression of 20,843 genes at T=0 and T=4, and the IV tolerance. In order to identify genes which differential expression influence the degree of tolerance, the DEG was also correlated to the IV tolerance. Significance threshold was set at p-value $< 5^{e}$-07.

Enriching the eQTL loci using IV cytokine response and tolerance data

In order to integrate the genetic variation associated with inter-individual gene expression, the eQTL loci were intersected with the cytokine data. The eQTL SNPs from the genome-wide significant eQTLs identified were extracted and associated with the IV cytokine response and tolerance. For the resulting ecQTLs and etQTLs a combined significance threshold was applied of a p-value < 5^{e}-08 for the eQTLs and an FDR < 0,05 for the cytokine- or tolerance QTLs. To identify the functionality of the QTL associated genes, EnrichR v3.2 was used to reveal the biological processes and pathways in which the genes were involved **[39]**.

Results

In this study the genotype, RNA-seq and clinical data from the 100LPS study was analyzed to identify transcriptome profiles or (e)QTLS that can be associated with the degree of inflammation upon first, and endotoxin tolerance upon second LPS challenge in healthy individuals.

Cytokine responses upon the first and second LPS challenge

Intravenous administration of LPS resulted in a profound increase in concentrations of pro-inflammatory cytokines TNF, IL-6, IL-8, IP-10, MIP-1a, MCP-1 and G-CSF as well as anti-inflammatory cytokines IL-1RA and IL-10 in all 100 subjects on both LPS challenge days (Figure 2A). The response upon second LPS challenge was severely blunted for all cytokines compared to the first challenge (median $\log_2$ fold change in AUC ranging from -2.8 for G-CSF to -0.3 for MCP-1, all $p < 0.0001$), indicative of endotoxin tolerance (Figure 2B).

Identifying genome-wide variants affecting cytokine production and endotoxin tolerance in response to LPS-challenge

3,333,357 and 3,311,421 genetic variants were mapped with IV cytokine production and endotoxin tolerance, respectively, with correction for gender and use of oral contraception (both factors were shown to influence cytokine responses in previous analyses, data not shown). This resulted in the identification of 3 significant cQTLs and 6 tQTLs. Annotating the QTLs revealed 7 associated genes with the cQTLs and 7 associated genes with the tQTLs (Table 1).

Interestingly, 7 out of the 14 associated genes are long noncoding RNA (lncRNA) genes which, in recent years, are increasingly being identified as key regulators of immunity and gene transcription during the inflammatory response. Functional annotation of these genes using EnrichR revealed their involvement in various biological pathways, including ATR activation (*HUS1*); MAPK cascade pathway (*MAP3K2-DT*); IL-4 regulation of apoptosis and IL-2 signaling (*TIMP2*); cytochrome p450 pathway (*CYP27C1*); and a member of the RAS Oncogene family (*RAB28*). Each of these pathways is linked to the regulation of immune functions. This indicates an association of the cQTLs and tQTLs with immune-regulatory functionality.

Among the list of QTLs, we discovered one tQTL to be genome-wide significant, namely rs7576783 in relation to G-CSF tolerance (Figure 3A). This SNP was found to be in high LD

with rs11889461 (R^2 = 0.96 and D' = 1) which was the second most significant tQTL identified in the analysis, associated with IL-8 tolerance (Figure 3B). Interestingly, in addition to being in high LD, the SNPs were found to associate significantly with the tolerance of each other's respective cytokine. Rs7576783 had a p-value of 1,19^{e}-07 when associated with IL-8 tolerance, and rs11889461 a p-value of 9,54^{e}-08 when associated with G-CSF tolerance. This, in combination with the SNPs being in high LD with each other, suggested the presence of a singular locus associated with decreased endotoxin tolerance for G-CSF and IL-8. Said locus was found to associate with *AC104623.2* which is a novel transcript antisense to *FAM49a* which has previously been connected to T-cell lymphopoiesis in the zebrafish.

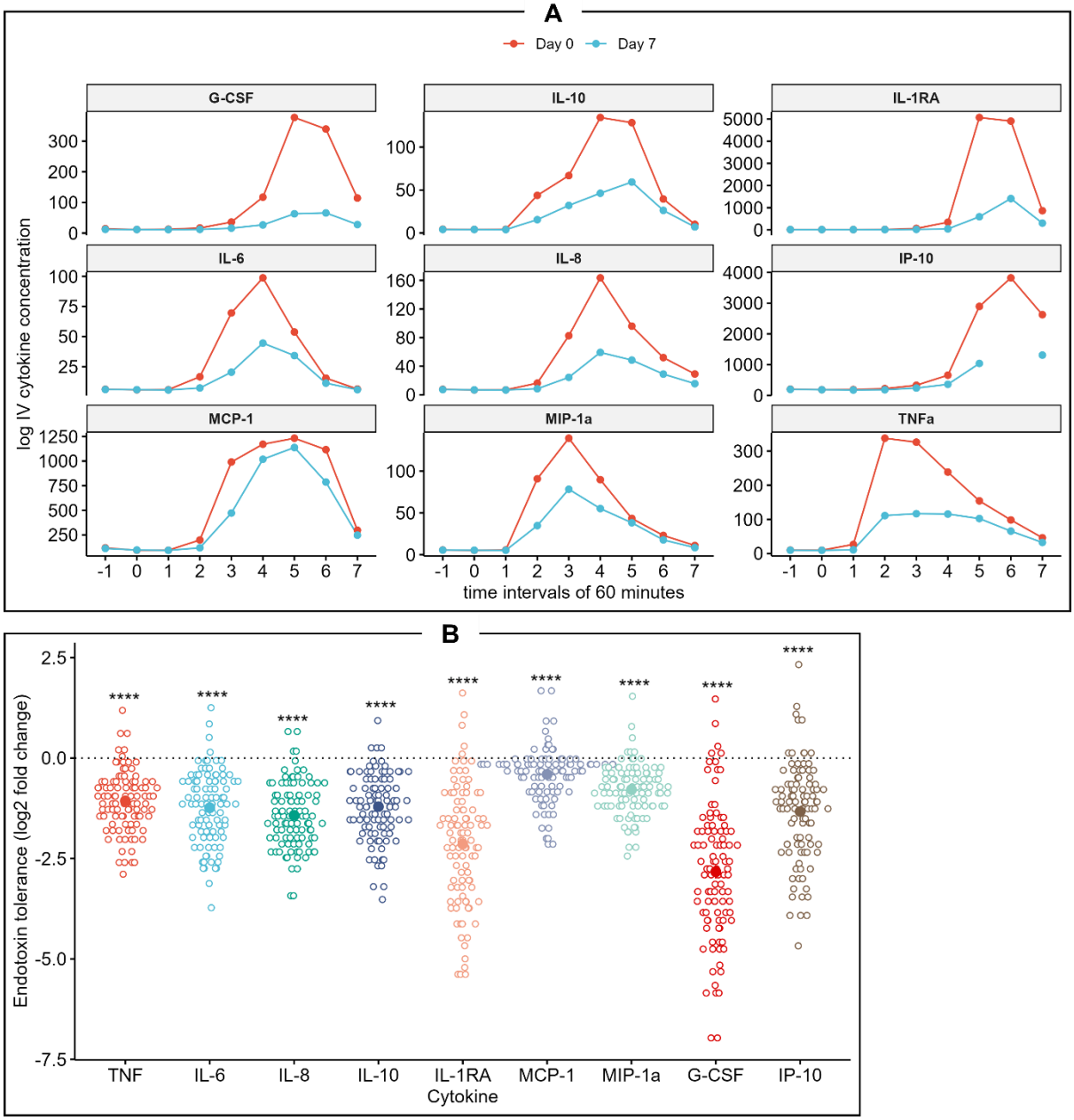

Figure 2. Cytokine response and tolerance development in the different compartments. A. Time-concentration curves of IV cytokine responses on both LPS challenge days. Data are displayed as geometric mean and IV cytokine concentration was log transformed. **B.** The extent of IV tolerance developed upon second LPS challenge. Tolerance values are expressed as log_2 fold change, with lower values indicating more pronounced tolerance. Tolerance values were log-transformed and tested for significant using a one-sample t-test against a hypothetical mean of 0 (****$p < 0.0001$).

Table 1. Summary table of SNPs associating significantly ($P < 5^e$-07) with the IV cytokine response and endotoxin tolerance.

QTL	SNP	Chr	Base Pair	Cytokine	Associated genes	P-value
Cytokine	rs73235328	4	13823366	IL-1RA	*RP11-341G5.1*[a], *BOD1L*[b], and *RAB28*[f]	2.13^e-07
Cytokine	rs2404668	5	79134949	MIP-1a	CTC-431G16.2[b]	2.67^e-07
Cytokine	rs10261383	7	47799423	TNF	*HUS1*[a] , *PKD1L1*[a], and *LINC00525*[d]	3.00^e-07
Tolerance	rs7576783	2	16703441	G-CSF	*AC104623.2*[b, e]	3.17^e-08
Tolerance	rs11889461	2	16700727	IL-8	*AC104623.2*[b, e]	7.21^e-08
Tolerance	rs7327173	13	22619013	IL-8	*LINC00540*[a]	2.81^e-07
Tolerance	rs34689155	2	127935156	MCP-1	*CYP27C1*[b, c], *MAP3K2-DT*[f]	2.29^e-07
Tolerance	rs4789932	17	76924275	MIP-1a	*TIMP2*[b]	1.04^e-07
Tolerance	rs1772980	6	2499839	MIP-1a	*RP1-80B9.2*[b]	2.26^e-07

[a]Gene shown in which the QTL is located.

[b]The closest gene to the QTL is shown

[c]QTL SNP is in linkage disequilibrium with a 3`-UTR variant within that gene.

[d]QTL SNP is in linkage disequilibrium with an intronic variant within that gene.

[e]QTL SNP is in linkage disequilibrium with a variant located within that gene.

[f]Expression QTL results show a correlation between QTL SNP and the expression of that gene.

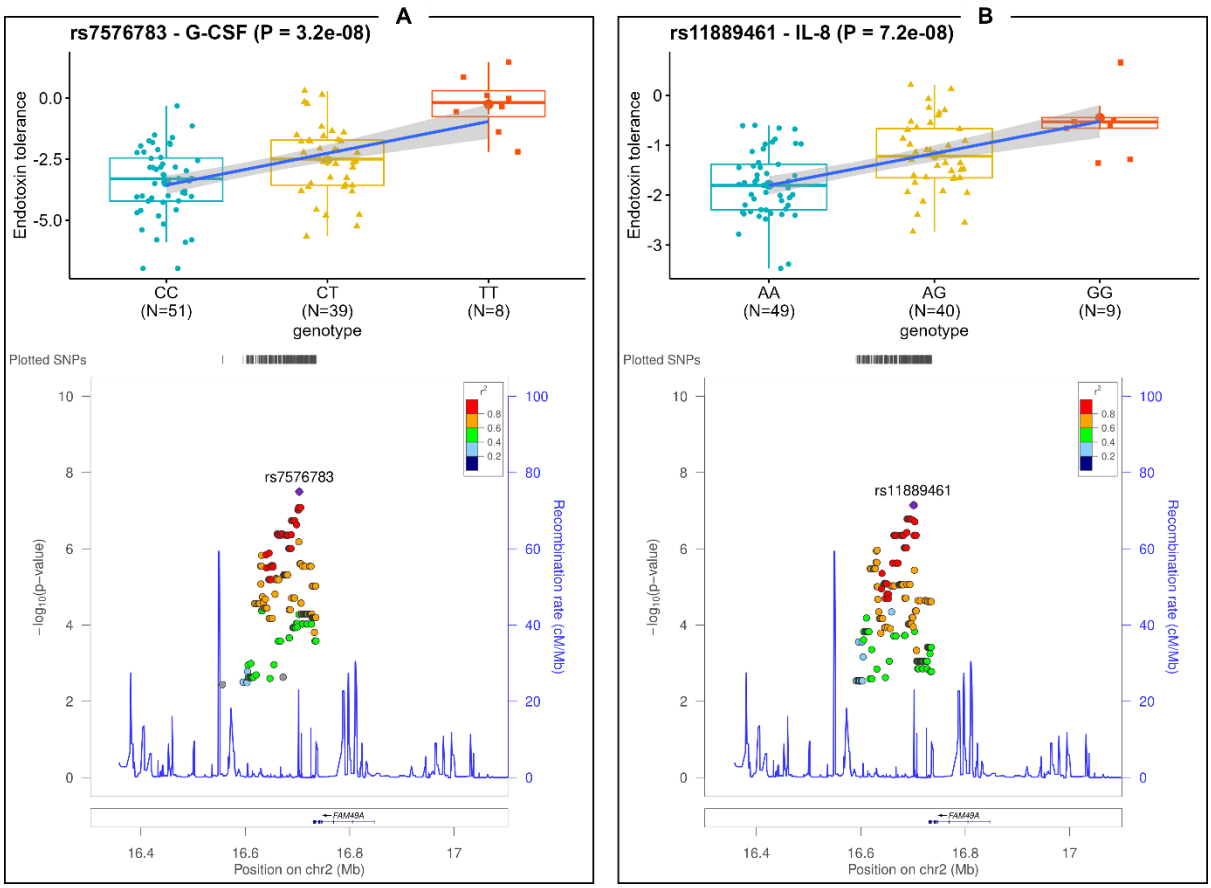

Figure 3. Region association plots and boxplots for tolerance QTLs. A-B. Regional association plots at the respective tQTL loci. Regional plots are indexed around the tQTL SNP and the corresponding P-values (as -$\log_{10}$ values) of all SNPs in the region were plotted against their chromosomal position. The boxplots display the genotype-stratified endotoxin tolerance (expressed as log2 fold change between the cytokine response on day 7 and the cytokine response on day 0) for the tQTL respective cytokine.

Correlating gene expression with cytokine response revealed genes directly associating with IV endotoxin tolerance.

The differential expression analysis of monocyte RNA-Seq identified 17,094 genes that significantly differed between T=0 and T=4 based on adjusted p-value < 0.05. Applying an absolute log2 fold change cut-off of 2 resulted in 566 genes that were significantly differentially expressed between T=0 and T=4. (Figure 4A). Enrichment revealed the majority of the genes to be involved in immune-regulatory pathways. The most significant pathways were the signaling pathways for IL-4, IL-10, and IL-13; classical antibody-mediated complement activation; chemokine receptors binding chemokines; and neutrophil degranulation. In addition to the many immune-related pathways, the genes were also found to be involved in many cell-metabolism, endothelial and cell-cycle/apoptosis pathways.

Correlating the IV cytokine AUCs with gene expression revealed no significant correlation at T=0, T=4 and DEG. Correlating IV tolerance with the gene expression at T=0 revealed no significant correlation. However, a total of 11 genes showed significant correlation at T=4 with tolerance (Table 2). Of these genes, increased expression of the known immune gene *IRAK3* was correlated with lower tolerance levels for IL-10 (Figure 5A). Additionally, *REEP5* was found to inversely correlate with the degree of tolerance for IL-10 (Figure 5B). *ENTPD1* was the only gene correlating with the tolerance of more than one cytokine, namely MIP-1a and TNF. Intriguingly, these correlations were the most significant ones identified in the analysis with p-values of $1{,}10^{e}$-09 and $1{,}40^{e}$-09 for MIP-1a and TNF respectively (Figure 5C). Interestingly, 7 of the 10 genes identified at T=4 were genes that correlated with tolerance for IL-10.

A significant correlation for 8 genes was observed between the DEG and IV tolerance. Two genes from the Kinesin Family, *KIF2a* and *KIF3c* were especially of interest. Their upregulation significantly correlated with increasing TNF tolerance levels. Moreover, we also found *KIF3c* expression at timepoint T=4 to significantly correlate with TNF tolerance. Associating the expression of *KIF3c* with the genetic variation identified a genome-wide DEG eQTL ($P = 1^{e}$-08) with SNP rs8051174 (Figure 5D). These findings suggest that expression of *KIF3c* is influenced by inter-individual differences in genotype for rs8051174, which in turn affects TNF tolerance.

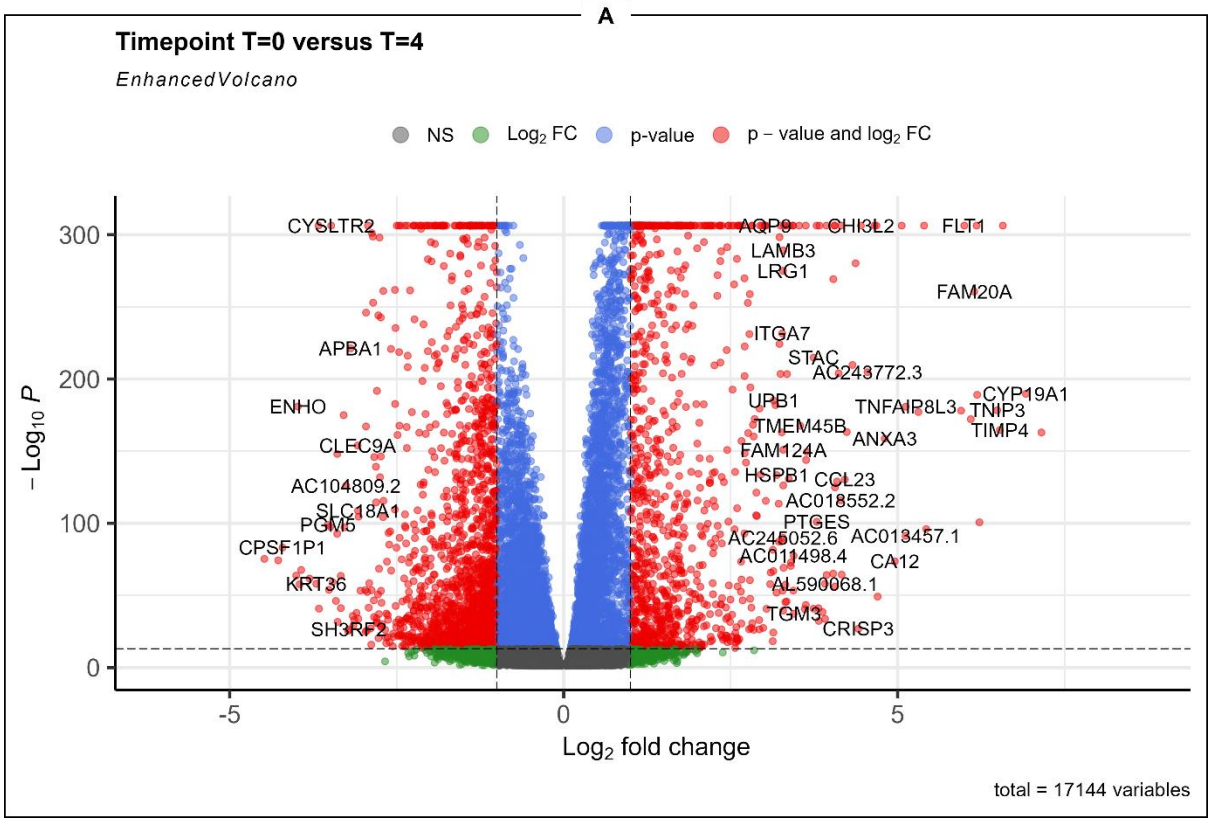

Figure 4. Volcano plot of differentially expressed genes between T=0 and T=4. A. Volcano plot of DGE: Timepoint T=0 versus T=4. X-axis displays the P-values as -Log_{10} and the y-axis displays the Log2 fold change of gene expression. Genes that were identified to have an absolute log2 fold change exceeding 3 were labelled.

Table 2. Summary table of genes correlating significantly (P < 5^{e}-07) with IV endotoxin tolerance development.

Timepoint	Gene	Cytokine	P-value	R correlation coefficient
T=4	*KIF3c*	TNF	1,60^{e}-07	-0,50
	IRAK3	IL-10	3,20^{e}-07	0,52
	GALE	IL-10	9,20^{e}-09	-0,54
	REEP5	IL-10	8,20^{e}-08	-0,51
	ENTPD1	MIP-1a	1,10^{e}-09	0,57
	ENTPD1	TNF	1,40^{e}-09	0,56
	HMG20a	IL-10	7,70^{e}-08	0,51
	CTBP1	IL-10	1,70^{e}-07	-0,50
	PNMA1	TNF	1,20^{e}-07	-0,50
	RASA3	IL-10	2,30^{e}-07	-0,49
	AL357060.1	IL-10	2,20^{e}-07	0,49

DEG	*KIF2a*	TNF	1,90^{e}-08	-0,53
	KIF3c	TNF	1,20^{e}-07	-0,50
	LDLRAP1	TNF	2,10^{e}-07	-0,50
	RHOBTB1	TNF	4,00^{e}-08	-0,52
	TNS1	MIP-1a	1,80^{e}-07	-0,50
	AL359644.1	MIP-1a	2,40^{e}-07	-0,49
	AC139713.2	TNFa	4,90^{e}-07	-0,48
	AC008669.1	IL-1RA	1,20^{e}-07	-0,50

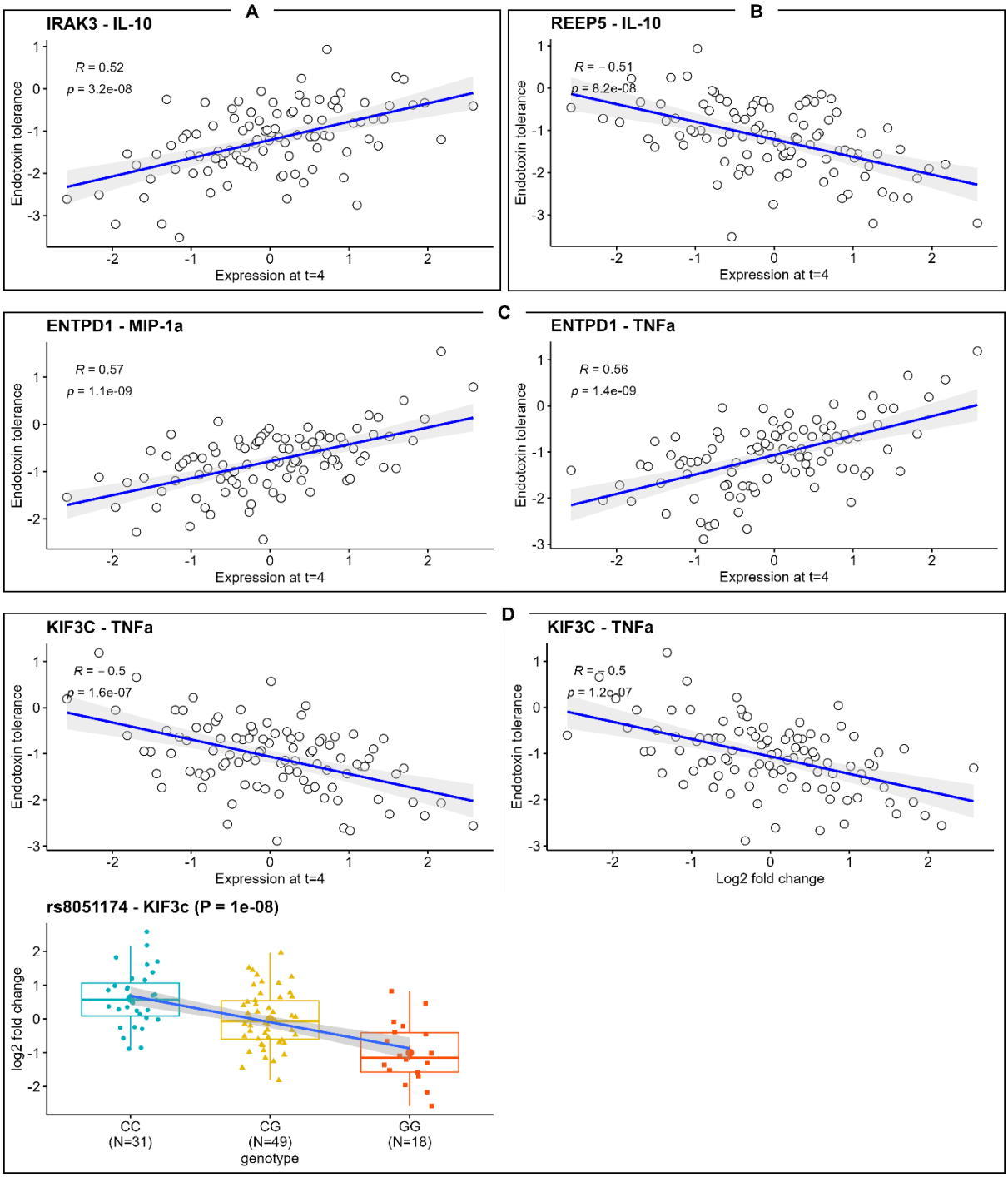

Figure 5. Correlations between IV endotoxin tolerance and expression at T=4. A. Correlation between *IRAK3* expression and IL-10 endotoxin tolerance (R = 0,52; P-value = 3,2^{e}-8). **B.** Correlation between *REEP5* expression and IL-10 endotoxin tolerance (R = -0,51; P-value = 8,2^{e}-8). **C**. Correlation between *ENTPD1* expression, and MIP-1a and TNF tolerance (R = 0,57; P-value = 1,1^{e}-9 and R = 0,56; P-value = 1,4^{e}-9, respectively). **D.** Correlations between IV endotoxin tolerance and *KIF3C* expression at DEG and T=4 (R = -0,50; P-value = 1,6^{e}-7 and R = -0,50; P-value = 1,2^{e}-7, respectively). Association boxplot for rs8051174 genotype-stratified expression of KIF3C (eQTL p-value: 1^{e}-08).

Integrating genotype, expression and cytokine data to identify genetic components driving differential gene expression and cytokine response.

The genome-wide association study at T=0 and T=4 identified 4,629 and 3,739 genome-wide significant eQTLs, respectively. The genome-wide association study using the DEG identified 922 genome-wide significant DEG eQTLs. We enriched the eQTLs identified by re-associating the significant eQTL SNPs with the AUC of the different cytokines, identifying SNPs which are both an eQTL and a cQTL, leading to so-called ecQTLs. Enriching the eQTLs at T=0 identified no SNPs associating significantly with IV cytokine AUC, while enriching the eQTLs at T=4 identified 7 ecQTLs influencing the expression of 7 genes and in turn 10 IV cytokine responses, mainly MIP-1a (Table 3). Enriching the DEG eQTLs lead to the identification of 14 ecQTLs that influence the expression of 15 genes and in turn 19 IV cytokine responses, especially for IL-6 and G-CSF.

The resulting genes were found to be involved in multiple biological processes such as regulation and mediation induction of (innate) immune response (*DDX41*); neutrophil degranulation (*DESG1*); hedgehog-signaling (*IFT52*); IL-4 and IL-13 signaling (BLC2); pathogenic E. coli infection (*TMEDI0* and *ABCF2*); innate immune system (*RNASET2*)*;* and infectious disease (*GATAD2B*) (Figure 6).

AT T=4, two ecQTLs were identified associating with more than one cytokine. The ecQTL for *GATAD2B* was found to significantly associate with upregulated expression and in turn with decreasing cytokine responses for G-CSF and IL-10 (Figure 7A). Furthermore, the ecQTL for *RNASET2* was identified to associate with downregulation in expression and subsequent increased cytokine responses of IL-6, MIP-1a, and TNF (Figure 7B).

Of the ecQTLs identified at DEG, *BCL2* and *DDX41* were found to be most readily annotated genes for immune-related pathways. Interestingly, both were significantly associated with the response of two cytokines each. The ecQTL associated with increased *BCL2* expression also associated with increased G-CSF and IL-8 responses (Figure 8A). Additionally, the ecQTL associated with decreased *DDX41* expression, increased IL-1RA and IL-8 responses (Figure 8B). Additionally, we found the ecQTLs for *TMED10* and *ABCF2* to also associate significantly with two cytokines but inversely when compared to *BLC2* and *DDX41*. Here, decreased expression resulted in decreased G-CSF levels (Figures 8C and 8D).

The ecQTL for genes *MKI67* and *MELK* was the only ecQTL identified for two genes. These genes have previously been reported to be involved in the RANKL regulation of the immune

response. When the expression of these genes was compared across the cohort, they were found to have similar up- or down-regulation for each participant which resulted in increased IL-8 and G-CSF cytokine response (Figure 8E).

Genetic variation in *ECE1* and *Lnc-LINS1-1* as predictors for endotoxin tolerance

The 4,629 and 3,739 genome-wide significant eQTLs at T=0 and T=4 were subsequently enriched with the IV endotoxin tolerance data in order to identify SNPs which are both an eQTL and a tQTL (etQTL).

At each timepoint T=0 and T=4, one significant etQTL was identified. Both etQTL SNPs were located within the gene of which the expression significantly correlated (Table 4). Rs12441485, an intronic variant identified at T=0, was located within the *LINS1* gene, regulating the expression of a sense intronic long-non-coding RNA gene of *LINS1*, namely *Lnc-LINS1-1*. The inter-individual differential expression of this gene at T=0 significantly influenced the endotoxin tolerance of IL-10 and G-CSF observed on day 7 (Figure 9A). At T=4, rs2072654 was identified as a significant etQTL, functioning as a 5`-UTR variant within the *ECE1* gene. Increased expression of *ECE1* at T=4 was found to result in increased tolerance levels for IL-6 and TNF (Figure 9B). In addition to timepoints T=0 and T=4, the DEG eQTLs were also enriched using the IV endotoxin tolerance data, but here, no significant etQTLs were identified.

Table 3. Summary table of eQTLs (P < 5^{e}-08) associating significantly with IV cytokine response (FDR < 0.05).

Timepoint	SNP	Cytokine	Gene	eQTL P	cQTL P	cQTL FDR
T=4	rs36043003	G-CSF	GATAD2B	2,47^{e}-08	1,79^{e}-05	0,05
		IL-8			1,92^{e}-05	0,03
	rs75783507	IL-6	IQGAP3	1,34^{e}-08	1,73^{e}-05	0,03
	rs5766289	IL-6	RNASET2	2,81^{e}-08	3,98^{e}-06	0,01
		MIP-1a			2,05^{e}-05	0,02
		TNF			1,67^{e}-05	0,04
	rs4820329	IL-8	SEC14L5	1,51^{e}-08	1,92^{e}-05	0,04
	rs265009	MIP-1a	RYR1	1,13^{e}-08	5,20^{e}-06	0,02
	rs9435839	MIP-1a	CCSAP	3,99^{e}-08	2,29^{e}-05	0,02
	rs11794029	MIP-1a	UAP1L1	1,84^{e}-27	5,63^{e}-05	0,04
DEG	rs9787347	IL-6	*WDR5B*	2,53^{e}-09	7,47^{e}-05	0,022
	rs6431621	G-CSF	*IFT52*	9,52^{e}-09	1,54^{e}-04	0,027
	rs13026372	IP-10	*CAMLG*	2,74^{e}-08	8,37^{e}-05	0,007
	rs999790	IL-6	*MYOSLID*	3,95^{e}-08	2,13^{e}-06	0,002
	rs9982079	G-CSF	*ABCF2*	8,43^{e}-09	9,37^{e}-05	0,027
		IL-8	*ABCF2*		1,98^{e}-05	0,008
	rs7620720	IL-1RA	*DDX41*	2,62^{e}-08	5,23^{e}-05	0,046
		IL-8	*DDX41*		1,06^{e}-04	0,031
	rs11241891	IL-10	*CTNND1*	3,60^{e}-08	3,09^{e}-06	0,003
	rs4467664	G-CSF	*MKI67*	1,15^{e}-08	1,42^{e}-04	0,026
		IL-8	*MKI67*		1,40^{e}-04	0,031
	rs4467664	G-CSF	*MELK*	4,92^{e}-08	1,42^{e}-04	0,026
		IL-8	*MELK*		1,40^{e}-04	0,031
	rs4897368	IL-6	*DEGS1*	4,22^{e}-08	2,04^{e}-04	0,042
	rs228436	G-CSF	*TNPO3*	1,77^{e}-08	2,45^{e}-05	0,014

rs645468	IL-6	*RASSF3*	1,52^{e}-08	7,11^{e}-05	0,022
rs7778583	G-CSF	*BCL2*	3,38^{e}-08	2,54^{e}-04	0,037
	IL-8	*BCL2*		2,92^{e}-06	0,003
rs12716535	G-CSF	*TMED10*	1,44^{e}-08	3,15^{e}-05	0,014
	IL-6	*TMED10*		2,53^{e}-04	0,042
rs2980754	IL-6	*AKAP11*	1,82^{e}-08	2,88^{e}-04	0,042

Table 4. Summary table of genes associating significantly (P < 5^{e}-08) with gene expression, and endotoxin tolerance (FDR < 0.05).

Timepoint	SNP	Cytokine	Gene	eQTL P	cQTL P
T=0	rs12441485	IL-10	*Lnc-LINS1-1*	2,95^{e}-08	6,11^{e}-05
		G-CSF			6,16^{e}-05
T=4	rs2072654	TNF	*ECE1*	1,10^{e}-09	2,75^{e}-05
		IL-6			4,36^{e}-06

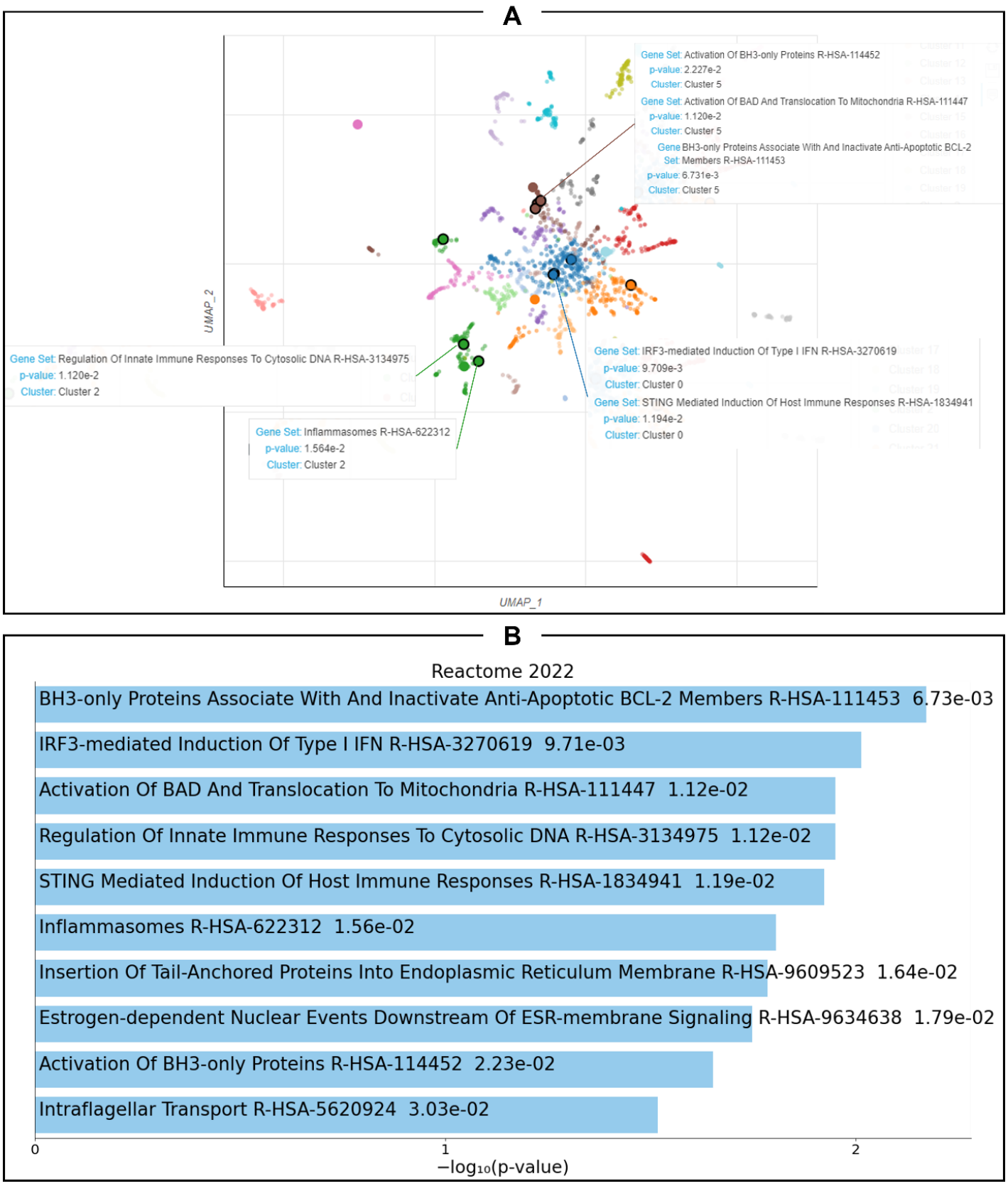

Figure 6. Cluster and bar chart of top enriched terms from the Reactome 2022 gene set library. A. Gene sets clustered based on Leiden algorithm. Terms with more similar gene sets are positioned closer together. The darker and larger the point, the more significantly enriched the term. **B.** The top 10 enriched terms for the input gene set are displayed based on the -log10(p-value), with the actual p-value shown next to each term.

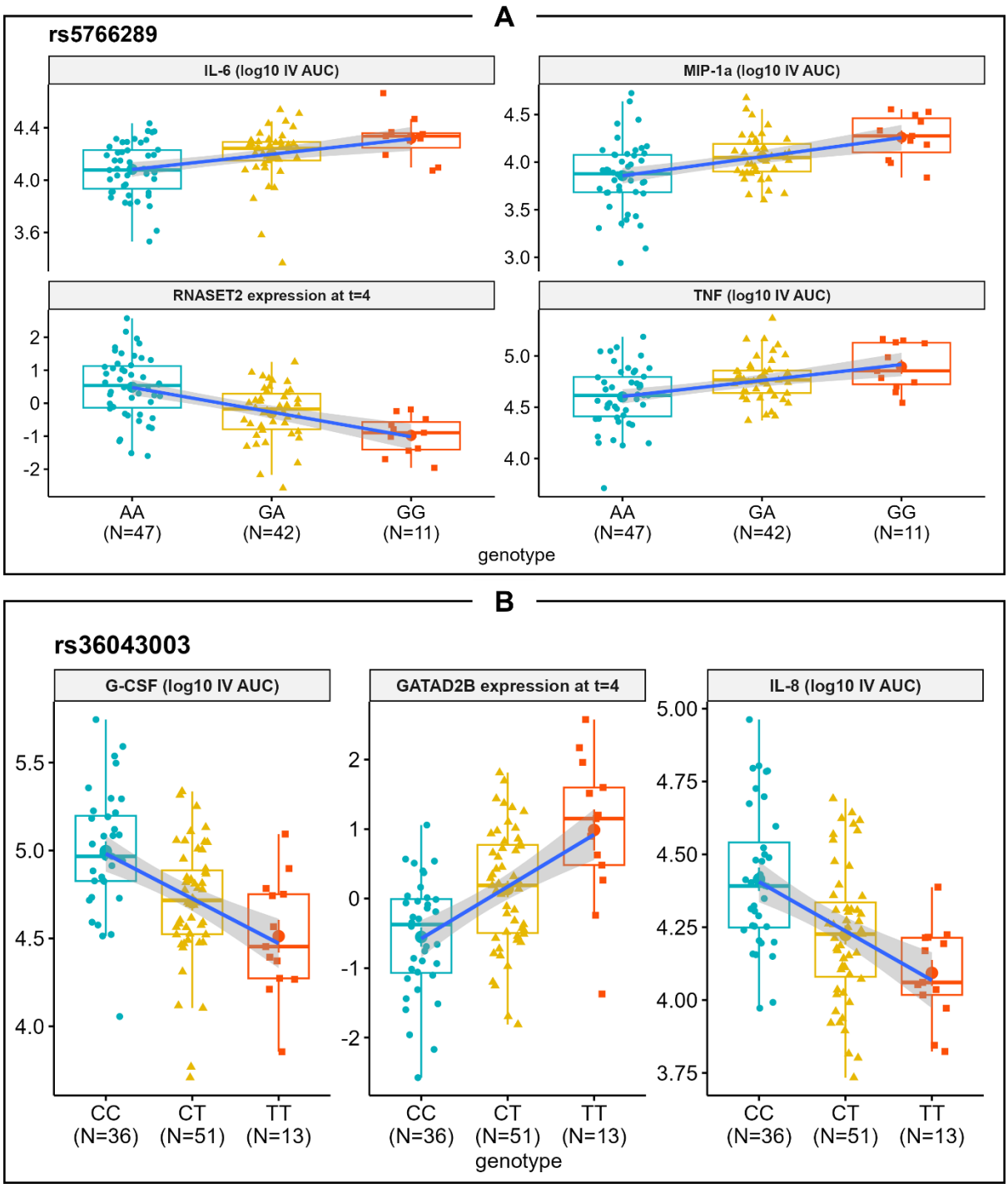

Figure 7. Association boxplots for the expression-cytokine QTLs identified at T=4. A-B. Regional association plots showing the genotype-stratified gene expression at T=4 and associated log10 IV cytokine AUC.

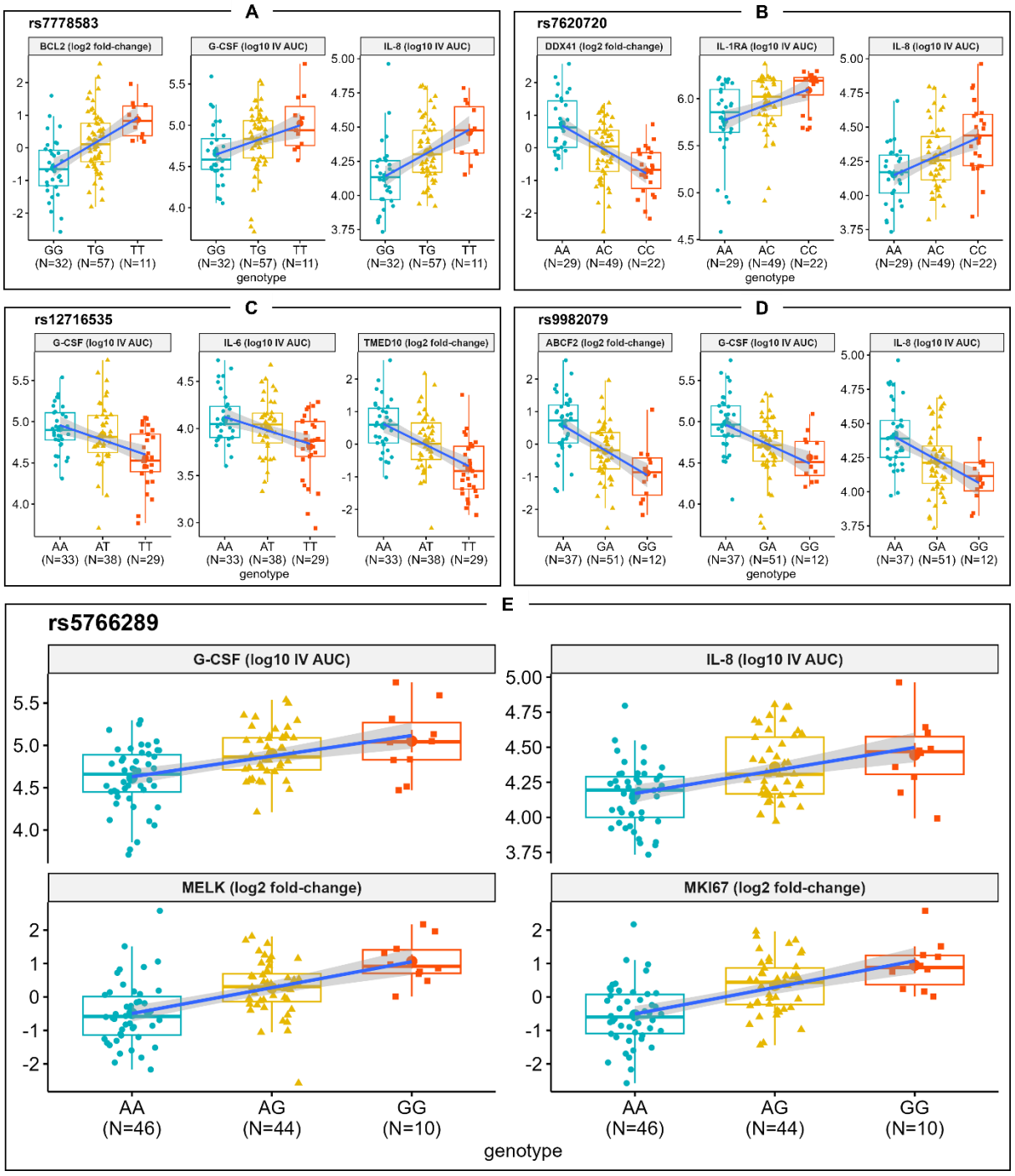

Figure 8. Association boxplots for the expression-cytokine QTLs identified at DEG. A-E. Regional association plots showing the genotype-stratified DEG gene expression and associated log10 IV cytokine AUC.

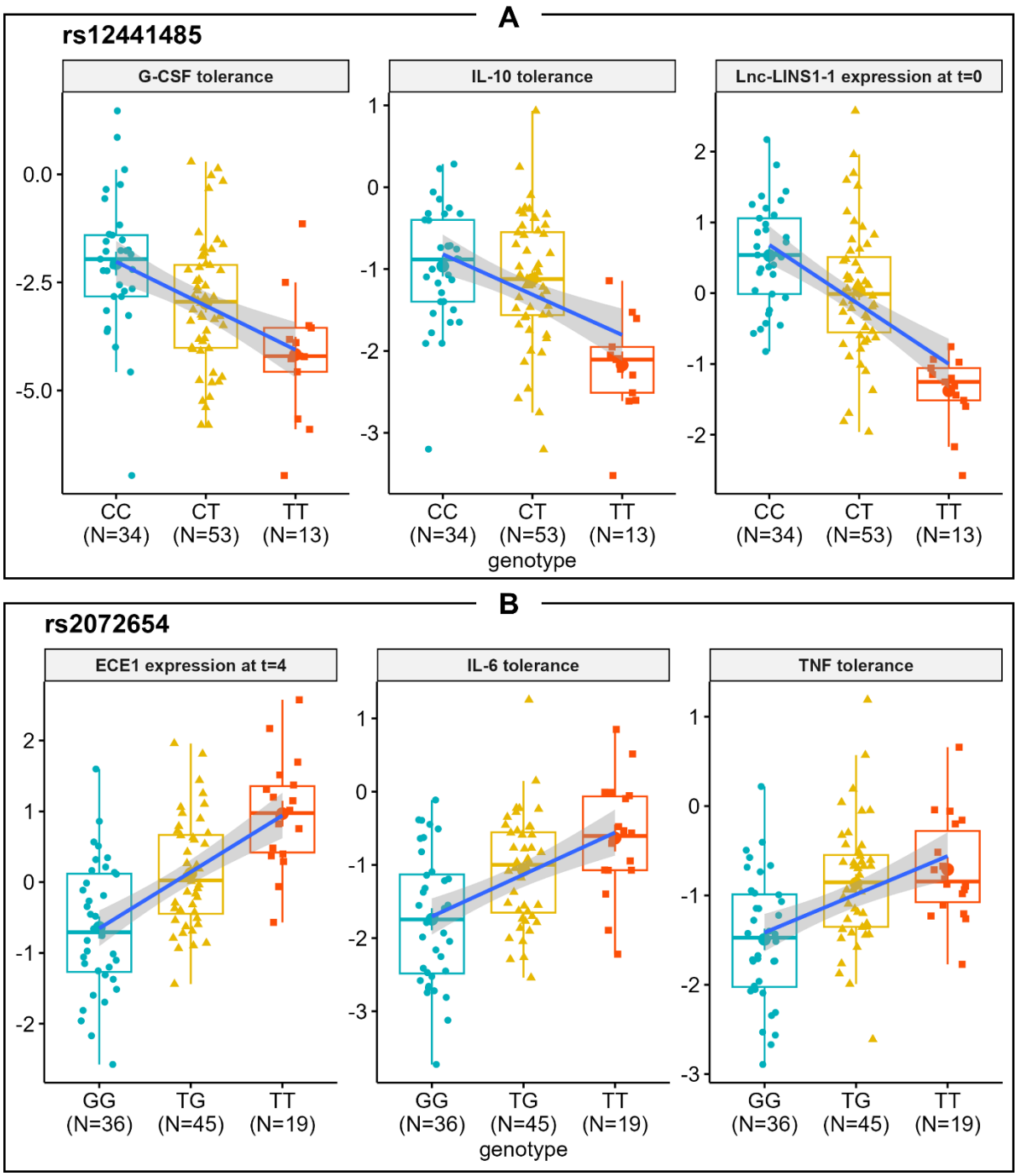

Figure 9. Boxplots for the identified expression-tolerance QTLs. A. Boxplots showing the genotype-stratified *Lnc-LINS1-1* expression at T=0 and associated G-CSF and IL-10 tolerance**. B.** Boxplots showing the genotype-stratified *ECE1* expression at T=4 and associated IL-6 and TNF tolerance.

Discussion

In recent years, the extreme heterogeneity and variety among sepsis patients has hampered therapeutic research into novel and effective sepsis treatments. These difficulties lie in the highly complex and heterogenous pathophysiology of sepsis. Therefore, a shift towards a more individualized treatment approach tailored to the immunological profile of the individual is warranted. In this study we employed the experimental human endotoxemia model and applied it to a homogenous cohort of healthy individuals. Subsequentially, we applied a complementary approach of integrating genotype, gene expression as well as *in vivo* cytokine and tolerance data to identify the genetic variation underpinning the inter-individual variation in immune responses and endotoxin tolerance.

In this study, many of the differentially expressed genes in monocytes upon LPS administration that displayed any type of correlation with tolerance were previously found to be associated with immune-related and endothelial pathways. In recent years, research has found that genetic loci associated with sepsis tend to prioritize immune-related and endothelial pathways **[16].** *ENTPD1*, whose differential expression directly correlated with the tolerance for two cytokines in the present work, was shown to be the rate-limited enzyme of a cascade leading to the generation of suppressive adenosine that alters CD4 and CD8 T cell activities **[40]**. Similarly, *IRAK3* modulates downstream signaling in the innate immune system **[41].** This resonates with findings made by Srinivasan et al, who identified an important paralog of *IRAK3, IRAK2* to associate with sepsis onset in infants **[42]**. The study also identified *CYP27A1* - a paralog of *CYP27C1*, which we identified as a tQTL. Additionally, 7 genes of the cytochrome p450 family were found to significantly differentially express between T=0 and T=4. This further implicates a role of signaling by *IRAK* and cytochrome p450 in sepsis.

The identified ecQTLs at T=4 were associated with *RNASET2* and *GATAD2B* expression and in turn IL-6, MIP-1a, TNF, G-CSF and IL-8 cytokine responses. SiRNA knockdown of *GATAD2B* has been shown to significantly reduce trans repression of *COX-2* and *IL-8*, suggesting that *GATAD2B* serves as an important mediator of P_4-PR suppression of proinflammatory genes **[43]**. *RNASET2* is directly involved in the immune response against Gram-positive bacteria **[44]**. Additionally, *RNASET2,* and ribonucleases in general, have been shown to be key players of the host immune response **[45]**. The DEG ecQTLs were associated with *BCL2* and *ABCF2* expression, and in turn G-CSF and IL-8 cytokine responses upon the first LPS challenge. Modulation of the *BCL2* family has previously been shown to block sepsis-

induced depletion of dendritic cells and macrophages, and blood *BCL2* levels in the first week of sepsis have been used to predict the mortality of sepsis patients **[46, 47]**. *ABCF1* – a paralog of *ABCF2* has been identified as a potential regulator of immunity and inflammation, and modulates sepsis mortality by promoting transition to the ER-phase **[48, 49]**. Using the results in this study we can further confirm the sepsis-related functionality of these genes and their involvement in (sepsis) cytokine responses. Additionally, considering that these previous findings were mostly identified in murine models of sepsis, our findings translate the functional implications of these genes to a human model.

Identification of tQTLs revealed the presence of a single locus associated with G-CSF and IL-8 tolerance. This locus was shown to associate with *AC104623.2* which is a novel transcript antisense to *FAM49a*. In the zebrafish this gene has been shown to modulate the PTEN pathway inhibiting T-cell differentiation **[50]**. Further research has found that the Fam49 protein family represents a new class of Rac1 interactors and observed significant reductions in CD4 and CD8 thymocytes as well as peripheral T cells in *Fam49b*-KO mice **[51, 52]**. Some implications of Fam49 have been found in *in vitro* HEK293T cell lines, but no further functional translation has been made to humans **[53]**. Here we show for the first time that *FAM49a* plays a role in the human immune system and development of endotoxin tolerance, in turn making it a potential biomarker and/or therapeutic target for sepsis-induced immunoparalysis.

Many of the results in this study implicate long-non-coding RNAs (lncRNA) in both cytokine responses upon the first LPS challenge and endotoxin tolerance. In recent years, lncRNAs have been shown to regulate both (post)-transcriptional and (post)-translational levels in a variety of ways **[54]**. Especially in the immune system, lncRNAs represent key regulators of the innate, adaptive, and humoral immune system and have been shown to play a role in sepsis **[55-57]**. Expression of the lncRNA *lnc-LINS1-1* at T=0 was shown to be regulated by variation in rs12441485 and identified as an etQTL that is a significant predictor for IL-10 and G-CSF tolerance. The *LINS-1* protein is a known modulating factor for the Wnt-β-Catenin signaling pathway, which is crucial in immune cell modulation, immune evasion and even playing a distinct role in sepsis **[58-60]**. While much is known about *LINS-1*, it has not yet been implicated in sepsis and sepsis-induced immunoparalysis. The results in this study show that the inter-individual genetic variation and expression in this gene may significantly impact endotoxin tolerance observed in healthy individuals. However, expression levels at T=0 (i.e. at baseline, before induction of inflammation) may not provide much clinical value in acutely ill patients, such as those with sepsis, where baseline samples are hardly ever available.

In this respect, a second potential predictor for endotoxin tolerance namely *ECE1* expression at T=4 (so in the acute inflammatory phase) regulated by rs2072654, is of more interest. The etQTL was found to be associated with a varying degree of tolerance for TNF and IL-6. *ECE1* is abundantly involved in endothelial pathways which, as mentioned above, have been shown to be prioritized in sepsis genetic loci **[61]**. Furthermore, *ECE1* has been shown to affect the MAPK signaling pathway and up-regulation of *ECE1* is closely linked to the presence of chronic inflammation in humans **[62, 63]**. While not much is known about the involvement of *ECE1* in the immune system yet, we demonstrate that it is associated with the development of endotoxin tolerance and therefore possibly immunoparalysis.

This is the first experimental human endotoxemia study using a large cohort of more than 110 subjects employing combined genotyping and gene expression analyses. Additionally, in contrast to studies in sepsis patients, our study population is highly homogeneous and is also not affected by confounding factors due to the highly standardized nature of the experimental endotoxemia protocol. This way much of the highly complex and heterogenous pathophysiology of sepsis could be negated which hampered earlies studies attempting to develop novel therapy targets for sepsis. Several of the pathways and genes that were identified in previous research to associate with endotoxemia and sepsis in mice- and *ex vivo* studies were also found to play a role in humans *in vivo* in this study. This underlines their relevance and functionally implicates their role in the human immune system and sepsis. These results furthermore substantiate the novel results put forward by this study.

A limitation of this study is the relatively limited sample size to identify genetic associations. Therefore, for many SNPs, the heterogenous alternate allele (minor allele) and especially for the homogenous alternate allele (least allele) could not significantly be associated with expression or cytokine responses. To increase statistical power, we filtered on strict minor- and least allele frequencies which led to the exclusion of 6 million SNPs.

It is recognized that the immune response in early stage sepsis is characterized by hyperinflammation and in later stages by sepsis-induced immunoparalysis leading to high mortality and morbidity, emphasizing the need for personal medicine and tailored treatment to the profile of the individual. Therefore, based on the results of this study, two genetic markers stand out that may have value for the prediction of degree of inflammation for G-CSF and IL-8 in the early immune response. The ecQTL rs9982079 for increased DEG of *ABCF2* and

decreased cytokine responses-, and ecQTL rs778583 for decreased DEG of *BCL2* and increased cytokines responses for G-CSF and IL-8

For the prediction of endotoxin tolerance and in turn sepsis-induced immunoparalysis, two genetic markers stand out. Firstly, a tQTL (rs7576783/rs11889461) within the *FAM49a* gene associated with tolerance for G-CSF and IL-8. Secondly, the etQTL rs2072654 that influenced *ECE1* expression during acute inflammation, which in turn influenced TNF and IL-6 tolerance.

These results may provide the first step towards early prediction of sepsis-induced immunoparalysis based on the genetic profile of sepsis patients. Furthermore, they may lead to the identification of new therapeutic targets. This opens up new avenues to develop new personalized immunostimulatory treatment strategies and increases prospects for future sepsis patients. For this to become a reality, further research, for instance functional studies using deletion or overexpression of *FAM49a, ECE1,* and additional studies into the three proposed SNPs are warranted.

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