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Functional and Genomic Architecture of *Borrelia burgdorferi*-Induced Cytokine Responses in Humans

Marije Oosting,[1,8] Mariska Kerstholt,[1,8] Rob ter Horst,[1] Yang Li,[2] Patrick Deelen,[2,3] Sanne Smeekens,[1] Martin Jaeger,[1] Ekta Lachmandas,[1] Hedwig Vrijmoeth,[1] Mihaela Lupse,[4] Mirela Flonta,[4] Robert A. Cramer,[5] Bart Jan Kullberg,[1] Vinod Kumar,[2] Ramnik Xavier,[6,7] Cisca Wijmenga,[2] Mihai G. Netea,[1] and Leo A.B. Joosten[1,9,*]

[1]Department of Internal Medicine and Radboud Center for Infectious Diseases (RCI), Radboud University Medical Center, 6525GA Nijmegen, the Netherlands
[2]Department of Genetics
[3]Genomics Coordination Center
University Medical Center Groningen, University of Groningen, 9713GZ Groningen, the Netherlands
[4]Department of Infectious Diseases, University of Medicine and Pharmacy "Iuliu Hatieganu," 400012 Cluj-Napoca, Romania
[5]Department of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Dartmouth, NH 03755-1404, USA
[6]Center for Computational and Integrative Biology and Gastrointestinal Unit, Massachusetts General Hospital, Harvard School of Medicine, Boston, MA 02114, USA
[7]Broad Institute of MIT and Harvard University, Cambridge, MA 02142, USA
[8]Co-first author
[9]Lead Contact
*Correspondence: leo.joosten@radboudumc.nl
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SUMMARY

Despite the importance of immune variation for the symptoms and outcome of Lyme disease, the factors influencing cytokine production during infection with the causal pathogen *Borrelia burgdorferi* remain poorly understood. *Borrelia* infection-induced mono-cyte- and T cell-derived cytokines were profiled in peripheral blood from two healthy human cohorts of Western Europeans from the Human Functional Genomics Project. Both non-genetic and genetic host factors were found to influence *Borrelia*-induced cytokine responses. Age strongly impaired IL-22 responses, and genetic studies identified several independent QTLs that impact *Borrelia*-induced cytokine production. Genetic, transcriptomic, and functional validation studies revealed an important role for HIF-1α-mediated glycolysis in the cytokine response to *Borrelia*. HIF-1α pathway activation and increase in glycolysis-derived lactate was confirmed in Lyme disease patients. In conclusion, functional genomics approaches reveal the architecture of cytokine production induced by *Borrelia* infection of human primary leukocytes and suggest a connection between cellular glucose metabolism and *Borrelia*-induced cytokine production.

INTRODUCTION

Lyme disease is the most common human vector-borne disease in the United States and Western Europe, and it is transmitted through infected ticks (Oosting et al., 2016). The disease is caused by bacteria of the species *Borrelia burgdorferi* sensu lato, including *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* as the major causative species. The clinical picture of Lyme disease is highly variable, ranging from early localized disease resulting in inflammation of the skin around the tick bite (erythema migrans [EM]) to disseminated infections, such as neuro-borreliosis and carditis. Furthermore, chronic or persistent forms of the disease are seen, resulting in long-term inflammation of large joints or the skin. In *Borrelia*-infected tissue or cultures of human cells exposed to the pathogen, high levels of cytokines and chemokines, including IL-1β and IL-17, have been detected (Bachmann et al., 2010; Burchill et al., 2003; Kuo et al., 2011; Shin et al., 2007). A crucial step in the induction of cytokine production by monocytes or macrophages is the recognition of pathogens, which is mainly mediated by pattern recognition receptors (PRRs). In recent years, several PRRs have been described to recognize ligands of *Borrelia* microorganisms, resulting in the induction of specific cytokine responses. Toll-like receptor (TLR) 2 together with nucleotide-binding oligomerization domain (NOD) 2 were shown to play an important role in the induction of cytokines after *Borrelia* recognition, whereas TLR10 was shown to have an inhibitory role in the *Borrelia*-mediated immune responses (Oosting et al., 2010, 2011a, 2014).

A large diversity is also seen in the induction of host defense, such as antibody and cytokine responses, in *Borrelia*-infected patients. Earlier research has shown that genetic variation in patients with Lyme disease strongly affects cytokine responses, ultimately leading to variation in clinical outcome (Oosting et al., 2011b). Additionally, other studies reported that non-genetic host factors, such as age, gender, vitamin intake, nutritional status, or smoking habits, can influence susceptibility to infectious or inflammatory diseases (Fish, 2008; Franceschi and Campisi, 2014; Khoo et al., 2012; Libert et al., 2010).

Despite the importance of immune variation for the symptoms and outcome of Lyme disease, no comprehensive understanding of the genetic and non-genetic factors influencing the cytokine production induced by *Borrelia* is currently available. A missing link between unraveling the genetic factors predisposing to Lyme disease and the translation into therapeutic targets is the understanding of the functional consequences of disease-associated genetic variation. Several questions therefore arise: what is the overall pattern of cytokine responses after stimulation of human primary leukocytes with *Borrelia*, how is this impacted by genetic variation and non-genetic factors, and lastly, do these factors influence susceptibility and clinical outcome in patients?

To address these questions, we profiled *Borrelia*-induced monocyte-derived and T cell-derived cytokines in two large cohorts of 500 and 200 healthy individuals of Western European ancestry from the Human Functional Genomics Project (500 Functional Genomics Project: 500FG and 200FG cohorts). We delineated inter-individual variability of *Borrelia*-induced cytokine production in the context of other microbial challenges, as also described in studies in *Cell* by ter Horst et al. (2016) and Li et al. (2016b), and we identified genetic loci that regulate *Borrelia*-mediated immune responses. Regulatory pathways identified through this genomic approach were validated in immunologic and transcriptomic studies.

RESULTS

Borrelia-Induced Cytokine Production Clusters Together with Intracellular Bacteria
To determine whether *Borrelia* spirochetes induce a pathogen-specific cytokine profile, we exposed peripheral blood mononuclear cells (PBMCs) from 500 healthy volunteers to *Borrelia burgdorferi s.s.*, *Borrelia* mix (i.e., equal amounts of *B. burgdorferi*, *B. afzelii*, and *B. garinii*), *Staphylococcus aureus*, *Mycobacterium tuberculosis* (Mtb), or *Escherichia coli* (*E. coli*) for 24 hr and 7 days. Overall, while inducing strong IL-1β and IL-6 responses, *Borrelia* induced almost no TNF-α production (Figures 1A and 1E). This was not due to experimental problems, as substantial TNF-α production was seen after stimulation with LPS (Figure 1H). Next, we assessed the correlation between *Borrelia*-induced cytokine production and cytokines induced by other intra- (i.e., Mtb) or extracellular bacteria (i.e., *E. coli* or *S. aureus*). A strong resemblance was found between the monocyte-derived cytokine profile induced by *Borrelia* and those induced by *E. coli* and Mtb but very little with *S. aureus*-induced cytokines (Figure S1). A different pattern emerged for the T cell-derived cytokines; *B. burgdorferi* and *Borrelia* mix cluster together, producing significant IL-22 and IL-17 but far less IFN-γ compared to the other stimuli tested (Figure 1C). Nevertheless, the strongest correlation on T cell-derived cytokines after *Borrelia* stimulation was again seen with the intracellular bacterium Mtb (Figure 1D).

To detect the presence of high and low responders to *Borrelia* exposure, we analyzed the distribution of cytokine production. Only *Borrelia*-induced IL-1β production was found to follow a normal (Gaussian) distribution (Figure 1E), while the production of all other measured cytokines was skewed to the right (Figures 1E–1H). This may be partially explained by the substantial amount of low responders (cytokine production below detection limit) seen for TNFα, IL-17, and IFN-γ.

### The Role of Non-genetic Factors for *B. burgdorferi*-Induced Cytokine Production
It has already been known that lifestyle influences immune responses, specifically cytokine production upon exposure to pathogens (Franceschi and Campisi, 2014; Libert et al., 2010). To detect differences in cytokine production after *Borrelia* exposure, we analyzed several factors, including previous exposure to tick bites, gender, body mass index (BMI), smoking habits, age, and vitamin D serum levels using a linear mixed model. Previous exposure to tick bites did not influence *Borrelia*-induced cytokine production (Figure 2A) regardless of the number of bites (data not shown). Stratifying on gender, BMI, smoking habits, and levels of circulating vitamin D did not affect cytokine production either (Figure 2A). Of high interest, however, age was found to have an important impact on *Borrelia*-induced cytokines in T cells (Figure 2A). PBMCs of elderly individuals produced significantly less IL-22 and IFN-γ after 7 days of *Borrelia* exposure, while no age-dependent effect was observed on IL-17 concentrations (Figure 2B). The same pattern was seen for Mtb stimulation, further strengthening the resemblance between the immune responses to *Borrelia* and Mtb. To determine whether previous exposure to *Borrelia* alters cytokine production upon (re-)stimulation with *B. burgdorferi*, *M. tuberculosis*, *S. aureus*, or *E. coli*, we stratified healthy individuals from the 200FG cohort based on the presence or absence of antibodies (IgG or IgM) against *Borrelia* (Figures 2C–2F; Figure S2). No differences in cytokine production, either monocyte-derived or T cell-derived, could be detected between individuals having positive or negative serology to *Borrelia*.

Genome-wide Mapping Identifies *Borrelia*-Specific eQTLs
Genetic factors have been described to influence cytokine production by immune cells and are able to strongly influence clinical outcome in disease (Netea et al., 2012). Therefore, we assessed genetic factors that specifically influence *Borrelia*-induced cytokine production. Cytokine production quantitative trait loci (cQTLs) analysis was performed as described (Li et al., 2016a). To correct for multiple testing, we set the threshold for genome-wide statistical significance at $p < 5 \times 10^{-8}$. However, using this threshold, only two cQTLs achieved genome-wide significance: rs17615278, located in the non-coding RNA transcript *RP11-92J19.3*, and rs11103976, located in *MGAT4C*, which codes for a glycosyltransferase protein. Both of these expression QTLs (eQTLs) were found for *Borrelia burgdorferi*-induced, but not *Borrelia*-mix-induced, IFN-γ production (Figure 3A). To increase our sensitivity for eQTLs affecting *Borrelia*-mix-induced cytokine production, we also analyzed cQTLs with p values between 1×10^{-5} and 1×10^{-8} for IL-22, IFN-γ (Figures 3B and 3C), IL-1β, and IL-6 (Table S1). To rule out that these eQTLs were found by chance, we performed validation in a separate cohort of healthy individuals (200FG). 55 top cQTLs for *Borrelia*-induced cytokines (Table S1) were also analyzed in the 200FG cohort, and 54/55 could be replicated for at least one cytokine (nominal p value < 0.05), including the two genome-wide significant cQTLs.

Expression QTL Mapping Reveals Genes Involved in *Borrelia*-Induced Cytokine Production
To identify the causal genes influenced by the detected cQTL SNPs, we performed eQTL mapping using RNA sequencing
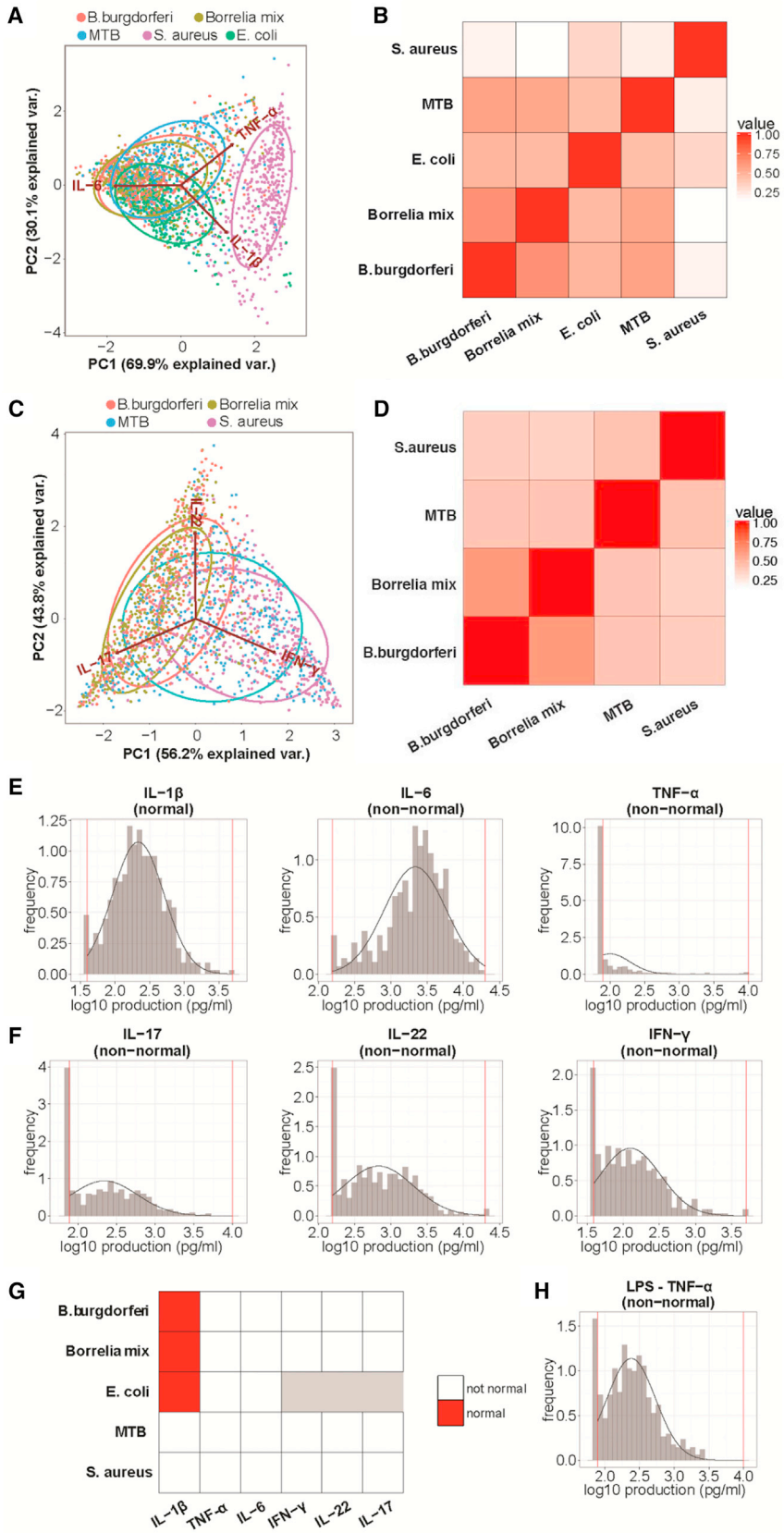
Figure 1. Similarities and Differences of *Borrelia* to Several Other Bacteria

(A) Principal Component Analysis (PCA) bi-plot based on the monocyte-derived cytokines (IL-6, TNF-α, and IL-1β). Each dot represents one individual's cytokine response against a particular stimulus; the colors indicate the different stimuli. The vectors indicate the importance of the original variables (cytokines) in creating the PCA axis system. The closer the points cluster along a vector, the higher the production for that cytokine.

(B) Correlation coefficients after 24 hr of stimulation with *S. aureus*, *M. tuberculosis*, *E. coli*, *Borrelia* mix, and *B. burgdorferi* for the monocyte-derived cytokines IL-1β, IL-6, and TNF-α, calculated using Spearman rank correlation.

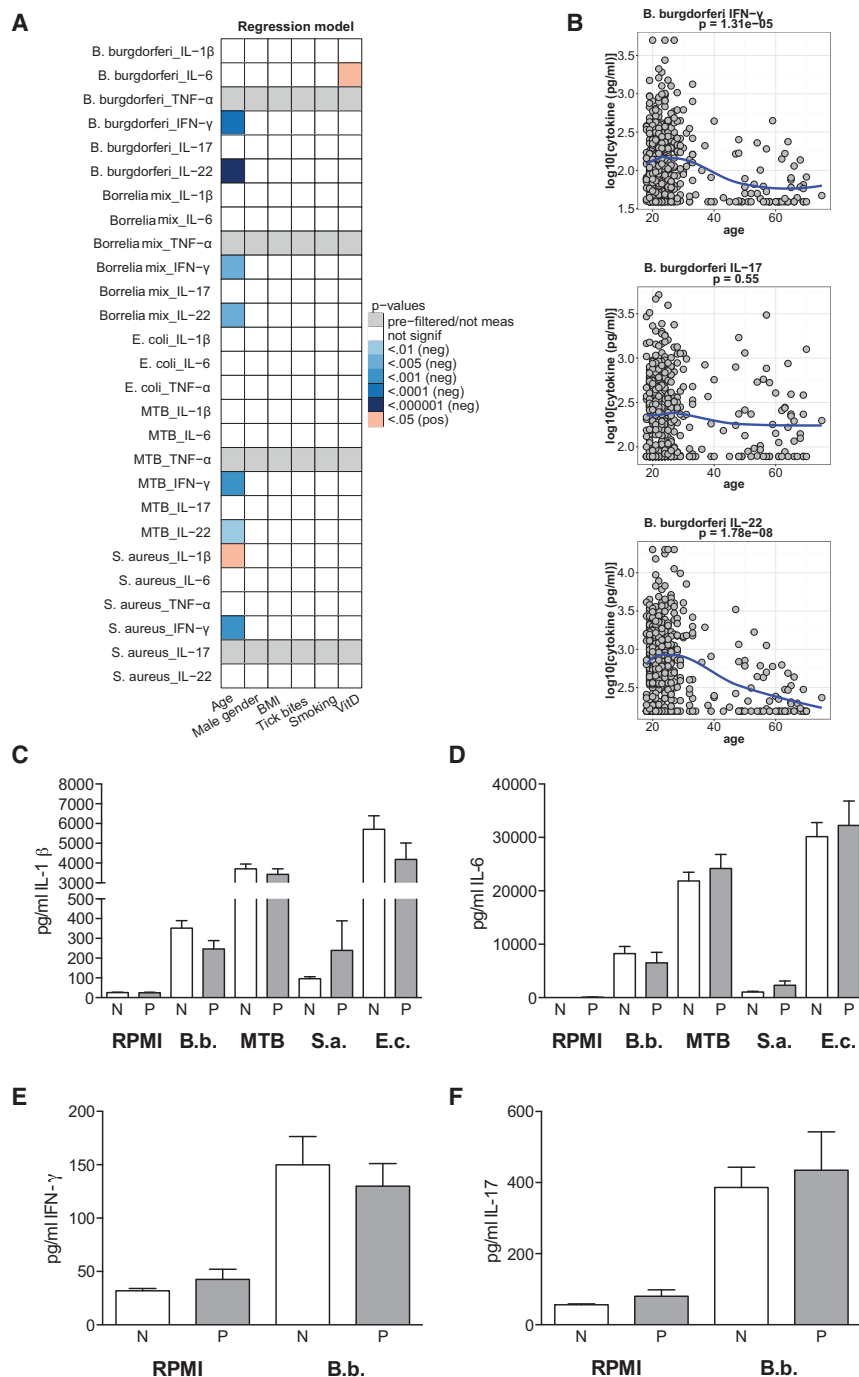(C) PCA bi-plot based on the T cell-derived cytokines (IL-17, IL-22, and IFN-γ).

(D) Correlation coefficients after 7 days of stimulation with *S. aureus*, *M. tuberculosis*, *Borrelia* mix, and *B. burgdorferi* for the T cell-derived cytokines IL-17, IL-22, and IFN-γ.

(E and F) Distributions of the monocyte-derived (E) or T cell-derived (F) cytokine responses for *B. burgdorferi*. The red lines indicate upper and lower thresholds of the ELISA measurement, and the black line indicates the best fitting theoretical Gaussian distribution.

(G) Heatmap showing the distribution of cytokine levels from the 500FG cohort. Red indicates normal distribution and white indicates non-normal distribution according to the Shapiro-Wilk normality test. Gray indicates that these cytokine distributions could not be tested.

(H) TNF-α production (pg/mL) after 24 hr stimulation with lipopolysaccharide (LPS).

A

Regression model

B. burgdorferi_IL-1β
B. burgdorferi_IL-6
B. burgdorferi_TNF-α
B. burgdorferi_IFN-γ
B. burgdorferi_IL-17
B. burgdorferi_IL-22
Borrelia mix_IL-1β
Borrelia mix_IL-6
Borrelia mix_TNF-α
Borrelia mix_IFN-γ
Borrelia mix_IL-17
Borrelia mix_IL-22
E. coli_IL-1β
E. coli_IL-6
E. coli_TNF-α
MTB_IL-1β
MTB_IL-6
MTB_TNF-α
MTB_IFN-γ
MTB_IL-17
MTB_IL-22
S. aureus_IL-1β
S. aureus_IL-6
S. aureus_TNF-α
S. aureus_IFN-γ
S. aureus_IL-17
S. aureus_IL-22

Age, Male gender, BMI, Tick bites, Smoking, VitD

p-values
pre-filtered/not meas
not signif
<.01 (neg)
<.005 (neg)
<.001 (neg)
<.0001 (neg)
<.000001 (neg)
<.05 (pos)

B

B. burgdorferi IFN-γ
p = 1.31e−05

B. burgdorferi IL-17
p = 0.55

B. burgdorferi IL-22
p = 1.78e−08

Figure 2. Effect of Non-genetic Factors on the *B. burgdorferi*-Induced Cytokine Production

(A) Heatmap depicting the statistical significance of the correlation between several non-genetic factors and cytokine production. Darker colors indicate higher levels of significance; red and blue cells correspond to positive and negative correlations, respectively. A gray cell means no analysis could be performed.

(B) Plot showing the correlation between age (x axis) and production of IFN-γ, IL-17, or IL-22 after 7 days incubation (y axis, pg/mL in log10 values).

(C–F) Production of IL-1β (C), IL-6 (D), IFN-γ (E), or IL-17 (F) by PBMCs from individuals with either negative (N) or positive (P) serology against *Borrelia*. Cytokines were measured after stimulation for 24 hr (C and D; n = 81 [N] and n = 31 [P]) or 7 days (E and F; n = 90 [N] and n = 41 [P]) with medium (RPMI), *B. burgdorferi* (B.b.), *M. tuberculosis*, *S. aureus* (S.a.), or *E. coli* (E.c.).

C

pg/ml IL-1β

N P N P N P N P N P
RPMI B.b. MTB S.a. E.c.

D

pg/ml IL-6

N P N P N P N P N P
RPMI B.b. MTB S.a. E.c.

E

pg/ml IFN-γ

N P N P
RPMI B.b.

F

pg/ml IL-17

N P N P
RPMI B.b.

was SNP rs55710213, which affects the mRNA expression of *HIF1AN*, encoding for the factor inhibiting HIF-1α (FIH-1), as seen in Figure 3D. This SNP significantly impacts IL-22 levels in PBMCs stimulated with *Borrelia* species while having less effect on Mtb and no effect at all on *S. aureus* stimulations (Figure 3E). Effect of this SNP was also seen, although weaker, on IL-17 production, but not in IFN-γ production (Figures 3F and 3G).
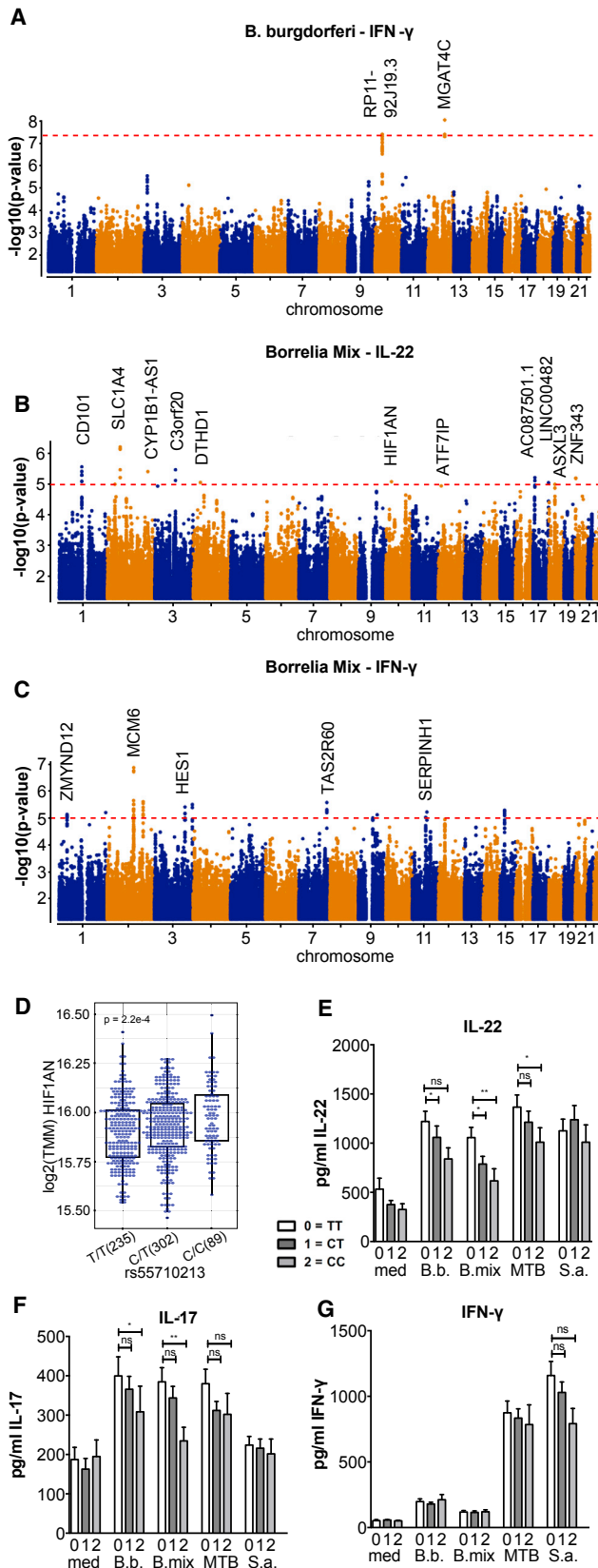
HIF-1α Regulation of Glucose Metabolism Is a Crucial Process for *Borrelia*-Induced Cytokine Production

We identified a SNP regulating the expression of the factor inhibiting *HIF-1α* as an important variable in *Borrelia*-induced cytokine production. HIF-1α is a transcription factor with a crucial role in regulating glucose metabolism, the response to hypoxia, and to antigenic stimulation (Sakamoto and Seiki, 2010; Scholz et al., 2016; Zhang et al., 2010). This suggests that cellular glucose metabolism may be important in *Borrelia*-induced cytokine production. To investigate this, we performed transcriptomic analysis on *Borrelia*-stimulated PBMCs from a previously published study (Smeekens et al., 2013). These data revealed significant upregulation of enzymes and transcription factors involved in the glycolysis pathway and downregulation of genes involved in the citric acid (TCA) cycle (Figures 4A–4C).

To confirm these findings, we performed functional assays to assess the activity of the glycolysis pathway and oxidative metabolism after stimulation. Corresponding to the transcriptomic data, PBMCs stimulated with *Borrelia* were shown to consume

data from 629 subjects in another separate cohort, the LL-DEEP cohort (Tigchelaar et al., 2015). We analyzed the top 20 cQTLs per *Borrelia*-induced cytokine and found 46 eQTLs (Table S1). Pathway analysis indicates that the eQTL genes are mainly involved in regulating general cellular responses, such as development and response to chemicals but also detection of bacterial lipopeptides (Table S2). However, enrichment analysis showed no specific pathway to be significantly overrepresented after correction for multiple testing. We therefore continued by assessing the eQTLs individually. The most interesting eQTL identified

A B. burgdorferi - IFN-γ

B Borrelia Mix - IL-22

C Borrelia Mix - IFN-γ

D

E IL-22

F IL-17

G IFN-γ

Figure 3. cQTL Analysis

(A–C) Manhattan plots showing the genome-wide QTL mapping results for *B. burgdorferi*-induced IFN-γ (A) and *Borrelia* mix-induced IL-22 (B) and IFN-γ (C). Horizontal dashed lines correspond to $p < 5 \times 10^{-7}$ (IFN-γ) or $p < 1 \times 10^{-5}$ (IL-22).

(D) Dot plot showing the expression levels, normalized by TMM method (trimmed mean of m values) (Robinson and Oshlack, 2010) of HIF1AN among the different genotypes for SNP rs55710213.

(E–G) Functional confirmation of SNP rs55710213 in the 500FG cohort. IL-22 (E), IL-17(F), and IFN-γ (G) production in pg/mL from PBMCs stimulated for 7 days. Values on the x axis represent the different genotypes; 0 = TT, 1 = CT, 2 = CC.

significantly more glucose and produce more lactate than unstimulated cells, leading to an increased extracellular acidification rate (Figure 4D). However, despite decreased transcriptional activity of genes in oxidative metabolism, no significant change was detected in oxygen consumption rate (OCR; Figure 4E). These findings indicate that *Borrelia* indeed modulates glucose metabolism in PBMCs, mostly by increasing the glycolysis pathway.

Previously, the PI3K/Akt/mTOR pathway was shown to be involved in initiating the metabolic switch toward aerobic glycolysis (Warburg effect) after stimulation (Gerriets and Rathmell, 2012; Weichhart et al., 2015). To test whether this is also the case after *Borrelia* stimulation, we analyzed protein expression of p-AKT and the downstream target of mTOR, p-4EBP1. As shown in Figure 4F, *Borrelia* stimulation increased activation of these signaling molecules, thereby supporting a role for the involvement of the PI3K/mTOR signaling pathway. To assess the role of the glucose metabolism pathways for *Borrelia*-induced cytokine production, we stimulated PBMCs with *Borrelia* in the presence of pharmacological inhibitors. Pretreatment of the cells with the glycolysis inhibitor 2-deoxyglucose (2-DG) drastically decreased production of IL-22 induced by *Borrelia* (Figure 4G). This effect was also seen for IL-1β, TNF-α, and IL-6 and the T cell-derived cytokines IFN-γ and IL-17 (Figures S3A–S3E). Conversely, complete shutdown of oxidative phosphorylation (OXPHOS) by the combined action of rotenone and antimycin A had no effect on cytokine production (Figure 4G, second panel). All inhibitors were checked for cytotoxicity by Annexin V/PI flow cytometry staining to rule out cell death as a confounding factor (Figure S4A).

Additionally, to analyze whether the PI3K/mTOR pathway is also involved in *Borrelia*-induced cytokine production, we treated the cells with the Akt-inhibitor wortmannin and the mTOR-inhibitors rapamycin and Torin1 and analyzed the production of IL-22 (Figure 4G). Interestingly, while mTOR inhibition significantly decreased IL-22 production, Akt inhibition actually showed a trend toward increasing IL-22 production. This may indicate uncoupling of these pathways in the case of *Borrelia* stimulation. Inhibiting HIF-1α significantly decreased production of IL-22 as well; however, this was accompanied by a substantial amount of cell death, as determined by Annexin V staining (Figure S4B). To further strengthen the association between HIF-1α-induced glycolysis and the immune response against *Borrelia*, we performed experiments on murine cells with an HIF-1α deficiency in the myeloid lineage. As seen in Figures 5A and 5B, cytokine production was reduced in HIF-1α knockout bone-marrow-derived macrophages (BMDMs) compared to
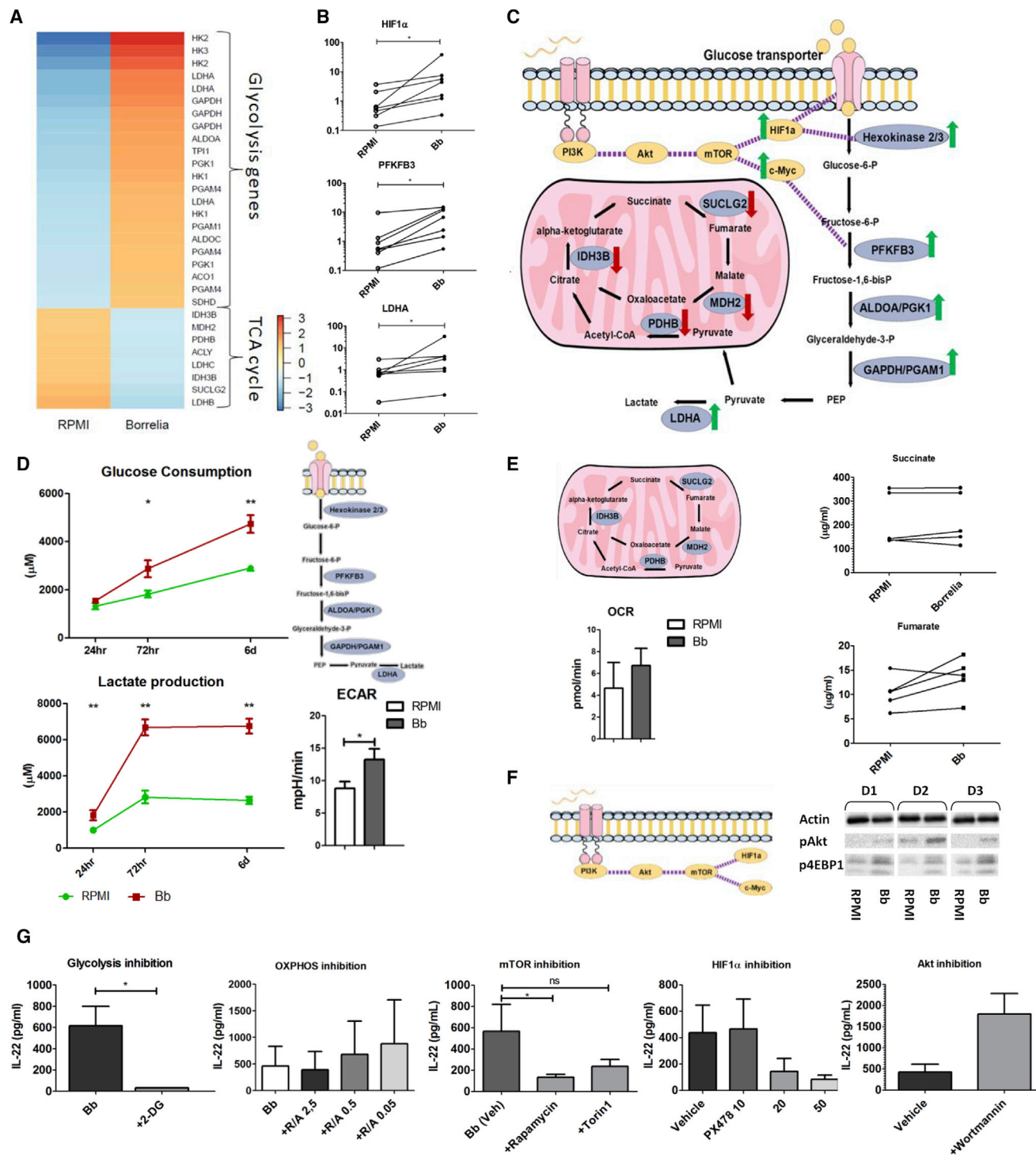
**Figure 4. *Borrelia*-Induced Changes in Glucose Metabolism**

(A) Heatmap of microarray data depicting genes in the glycolysis and TCA cycle pathway significantly affected by *Borrelia* stimulation in healthy PBMCs (n = 48).

(B) qPCR confirmation of a representative selection of genes in healthy PBMCs (n = 7) stimulated with *Borrelia*.

(C) Graphical representation of enzymes and transcription factors in central metabolic pathways, whose expression was significantly affected by *Borrelia* stimulation (green arrows, upregulation; red arrows, downregulation). Figure was designed in the MindtheGraph platform. *p < 0.05, paired t test.

(D) Functional analysis of glycolysis pathway activity measured by glucose consumption and lactate production after several time points and confirmed by measuring extracellular acidification rate (ECAR) in healthy PBMCs after 24 hr stimulation with *B. burgdorferi* (B.b.) (n = 6).

(legend continued on next page)
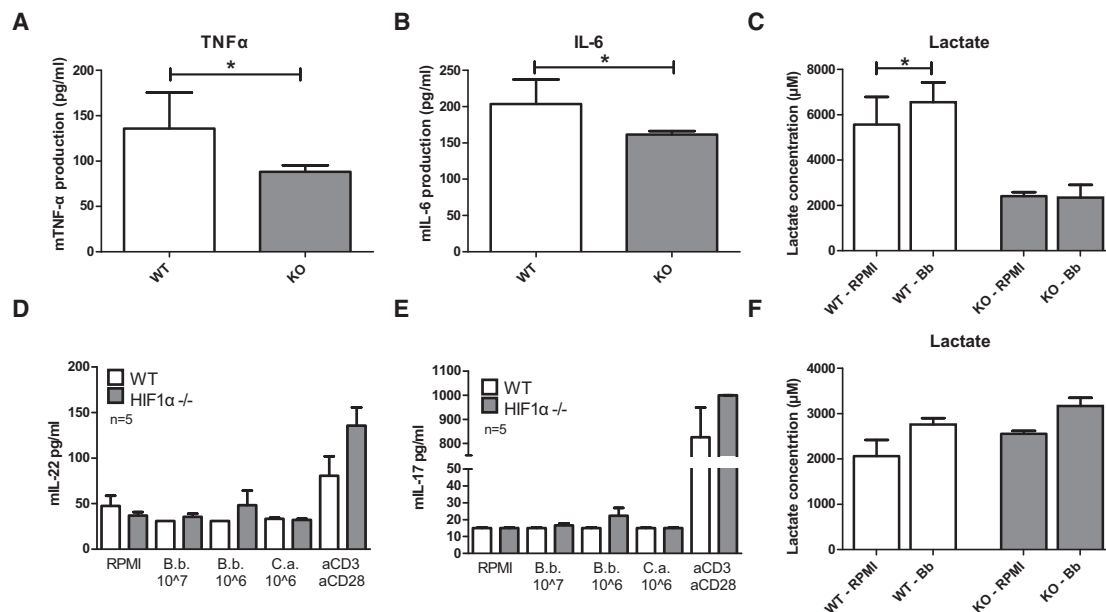
Figure 5. Borrelia Stimulation in HIF-1α⁻/⁻ Cells

Production of innate cytokines, TNF-α (A) and IL-6 (B), and lactate (C) was determined from cell culture supernatants of HIF-1α$^{-/-}$ murine bone-marrow-derived macrophages after 24 hr of stimulation with *Borrelia burgdorferi* (10^6 spirochetes/mL). Production of T cell-derived cytokines, IL-22 (D) and IL-17 (E), and lactate (F) was determined in cell culture supernatants from murine spleen cells with an HIF-1α knockout in the myeloid lineage (n = 5) after 6 days of stimulation with *Borrelia burgdorferi* (10^6 and 10^7 spirochetes/mL, respectively), *Candida albicans* (C.a.), or anti-CD3/anti-CD28 antibodies.

wild-type (WT) cells after 24 hr stimulation with *Borrelia*. Correspondingly, lactate production was significantly lower in HIF-1α$^{-/-}$ BMDMs compared to WT cells (Figure 5C). These effects were not seen in spleen cells (Figures 5D–5F), which is not surprising, as these consist mainly of T cells, and the HIF-1α deficiency was restricted to cells of the myeloid lineage. All together, these data show that *Borrelia* strongly increases the glycolysis pathway in healthy PBMCs, predominantly through the mTOR/HIF-1α pathway, and that activation of this pathway is essential for cytokine production.

Enhanced HIF-1α Expression and Lactate Production in Patients with Early Lyme Disease

To determine whether HIF-1α-mediated glucose metabolism is also involved in patients, we analyzed PBMC transcriptomic profiles of 29 patients with physician-documented EM from a previously published study (Bouquet et al., 2016). These data corroborated our in vitro findings, with *HIF1A* mRNA being significantly upregulated in PBMCs at the time of diagnosis (T = 0) and 2 weeks thereafter (T = 2w). Correspondingly, *HIF1AN* was significantly downregulated at T = 0 (Figures 6A and 6B). After 6 months, the expression levels of both *HIF1A* and *HIF1AN* were normalized. In addition, other genes in the glycolysis pathway, such as *HK2*, *PFKFB3*, and *LDHA*, were also

significantly upregulated in acute Lyme patients (Figures 6C–6E). Finally, we determined the serum concentration of lactate, the end product of glycolysis, in a separate cohort of patients with recent onset Lyme disease. In line with the enhanced *HIF1A* and decreased *HIF1AN* expression, we showed that serum lactate is elevated shortly after *Borrelia* infection (Figure 6F). All together, these results underscore the involvement of glucose metabolism in immune cells for the host response against *Borrelia* infection.

DISCUSSION

Cytokines are a crucial component of the immune system, and they play an important role in both the antimicrobial host defense, as well as in the pathophysiology of immune-mediated diseases. This is also the case for Lyme disease, but little is known regarding the variability in these responses between individuals. The Human Functional Genomics Project investigates the variability of human immune responses in general, with a specific interest in cytokine responses. We report the overall impact of environmental (ter Horst et al., 2016) and genetic (Li et al., 2016b) host factors, as well as the microbiome (Schirmer et al., 2016) for modulating cytokine responses to microbial and non-microbial stimulation. In the present study, we focused

(E) Functional analysis of mitochondrial metabolism measured by oxygen consumption rate (OCR) and measurements of fumarate and succinate, two intermediates in the TCA cycle after 24 hr stimulation with *Borrelia*.

(F) Western blots of phospho-Akt and phospho-4E-BP1 after 2 hr stimulation with RPMI or *Borrelia*. Image was cropped.

(G) Effect of inhibition of the glycolysis pathway (n = 5), oxidative phosphorylation (OXPHOS) (n = 6), mTOR (n = 6), HIF-1α (n = 5), or Akt (n = 6) on *Borrelia*-induced IL-22 production. R/A, combination of Rotenone and Antimycin A (concentrations 2.5, 0.5, and 0.05 μM). *p < 0.05, **p < 0.01.
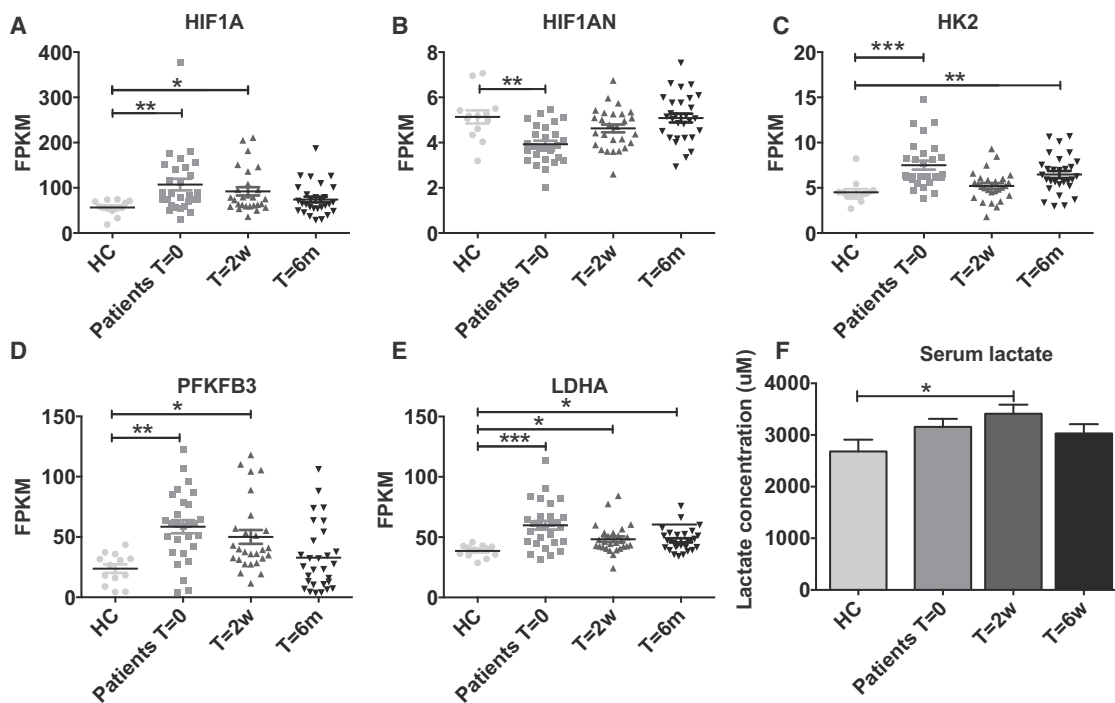
Figure 6. Transcriptomic Analysis of PBMCs from Lyme Disease Patients

(A–E) Gene expression in FPKM (fragments per kilobase million) of *HIF1A* (A), *HIF1AN* (B), *HK2* (C), *PFKFB3* (D), and *LDHA* (E) in healthy controls (HC; n = 13) versus EM patients (n = 28) at different time points. Data were obtained from a publicly available dataset (Bouquet et al., 2016), GEO: GSE63085.

(F) Serum lactate concentrations in a cohort of healthy controls (n = 22) compared to EM patients (n = 58) sampled at different time points. *p < 0.05, **p < 0.01.

on deciphering the functional and genetic architecture of the *Borrelia*-induced cytokine responses.

The general cytokine profile in PBMCs stimulated with two preparations of *Borrelia* and several other pathogens was assessed. It should be noted that this model reflects the systemic response to the bacterium rather than the initial inflammatory response, which occurs locally in the skin. No large differences were seen between the responses to *Borrelia* alone or the *Borrelia* mix. When comparing with other bacterial stimuli, the *Borrelia*-induced cytokine profile most closely resembled that of Mtb. Biologically, this is an important observation, as Mtb and *Borrelia* are both recognized by a combination of both membrane-bound (TLR2) and intracellular (NOD2) receptors, while the extracellular bacteria are largely recognized by membrane-bound receptors only (Kleinnijenhuis et al., 2011, 2012; Oosting et al., 2010, 2011a). The early cytokine responses seem therefore to follow a recognition receptor-oriented pattern. This is in line with the overall architecture of cytokine responses reported by Li et al. (2016a), which are pathogen centered, rather than immune pathway centered. Another important observation is the differential regulation of *Borrelia*-induced Th1 and Th17 cell-derived cytokine production: while high production of both IL-17 and IL-22 were observed, IFN-γ production remained low. The latter may be an important observation, as it may represent a strategy of the microorganism to evade host defense. Strong induction of IL-22, but not IL-17, by *Borrelia* has been previously reported (Bachmann et al., 2010). This difference may rely on the short exposure time in this previous investigation, as we recently demonstrated that

T cells, and not NK-cells, are the main source of IL-22 and IL-17, as well as IFN-γ, after *Borrelia* stimulation. A minimum of 4 days exposure to *Borrelia* in vitro is therefore necessary for induction of IL-17 in PBMCs (Buffen et al., 2016). The relevance of IL-17 and IL-22 production for the pathophysiology of Lyme disease is not fully understood, although both cytokines are reported to play a role in the persistent phase of the disease (Codolo et al., 2008; Kotloski et al., 2008). It is known that IL-22 is important in epithelial skin barrier function and therefore might play a role in dissemination of the spirochetes (Aujla and Kolls, 2009; Bachmann et al., 2010). Additionally, genetic variations affecting IL-22 production have been previously shown to affect susceptibility to tuberculosis (Zhang et al., 2011) or candidiasis (De Luca et al., 2013).

Next, we assessed the effect of non-genetic host factors on cytokine responses to *Borrelia*. These appeared to play a limited role, with the notable exception of age. Increasing age strongly decreased *Borrelia*-dependent IL-22 production, and this effect was more pronounced for *Borrelia* than for other stimuli (ter Horst et al., 2016). This may have important implications, as this could indicate higher susceptibility to Lyme disease in the elderly. It is already known that the incidence of Lyme disease shows a bimodal age distribution with a peak incidence in individuals aged 50–70 (Berglund et al., 1995; Fülöp and Poggensee, 2008; Nelson et al., 2015), though it is difficult to distinguish biological age-related factors from behavioral factors. Additionally, several disseminated Lyme disease symptoms, such as acrodermatitis chronica atrophicans (ACA), are known to be more prevalent in elderly individuals (Mullegger, 2004).

Surprisingly, neither previous exposure to tick bites, nor previous encounters with the spirochete, defined by the presence of *Borrelia* antibodies, influenced the cytokine response. Lastly, one of the most important aims of the present study was the identification of the genomic architecture of cytokine responses in immune cells upon stimulation with *Borrelia*. Therefore, a genome-wide association of eight million SNPs with *Borrelia*-specific induction of cytokines was performed, and cQTLs influencing both monocyte-derived and T cell-derived cytokines were identified. Most of these cQTLs encode for genes with unknown function for the cytokine responses to *Borrelia*, and follow-up studies are warranted to investigate their role. It should be noted that different eQTLs were found for *Borrelia burgdorferi*- and *Borrelia*-mix-induced cytokines, indicating that different pathways may be involved in the immune response against the different *Borrelia* species. This is also reflected by the fact that the different species are known to induce different symptoms (van Dam et al., 1993).

A very interesting link was identified between cellular glucose metabolism and *Borrelia*-induced cytokine production. One of the most significant eQTLs was found for a SNP regulating the transcription of *HIF1AN*, encoding for the factor inhibiting HIF-1α (*FIH-1*), which significantly affected *Borrelia*-induced IL-22 levels. HIF-1α is a transcription factor with a crucial role in regulating glucose metabolism (Sakamoto and Seiki, 2010; Scholz et al., 2016; Zhang et al., 2010). In line with this, *Borrelia* was found to induce a shift in cellular metabolism toward a Warburg effect: a switch from oxidative phosphorylation to aerobic glycolysis, mediated by the mTOR/HIF-1α pathway. This switch has been previously reported to be involved in cytokine production during activation of Th1 (Cham and Gajewski, 2005; Chang et al., 2013) and, more recently, also Th17 cells (Gerriets et al., 2015; Michalek et al., 2011) and NK-cells (Keating et al., 2016). Interestingly, although many previous studies showed the involvement of the PI3K/Akt pathway in activating mTOR, our data indicate that IL-22 production after *Borrelia* stimulation was Akt independent, yet highly dependent on mTOR. This indicates that mTOR is activated through an unknown alternative pathway. Although uncommon, similar findings have been previously reported (Brewer et al., 2007). Nevertheless, our in vitro data showed that the induction of glycolysis through the mTOR/HIF-1α pathway is essential for *Borrelia*-induced production of IL-22, as well as other cytokines. Furthermore, experiments in HIF-1α$^{-/-}$ bone-marrow-derived macrophages showed that both cytokine production and lactate production were decreased compared to WT cells. This strongly supports a role for HIF-1α-mediated glycolysis in *Borrelia*-induced cytokine production. Altogether, our data provides evidence that the glycolysis pathway is physiologically relevant in the human immune response. Our findings showing a stronger effect of the *HIF1AN* eQTL on IL-17 and IL-22 rather than on IFN-γ are supported by Michalek et al. (2011), showing a higher glycolytic rate in Th17 cells compared to Th1 cells, which could explain the higher sensitivity to changes in glycolytic flux. In addition, Gerriets et al. (2015) provided evidence for an important role for PDHK1, present in Th17 cells but not in Th1 cells, for glycolysis and inflammation. Also, transcriptional profiling of leukocytes of patients with Lyme disease resulted in significantly upregulated genes in the mTOR-HIF-1α and glycolysis pathway in white blood cells shortly after *Borrelia* stimulation. In line with these findings, we found that serum lactate was elevated 2 weeks after initial diagnosis of *Borrelia* infection. Furthermore, lactate levels in cerebral spinal fluid (CSF) were previously shown to be strongly increased in 63% of Lyme patients with facial palsy, while CSF lactate was normal in patients with facial palsy due to other infections (Kindler et al., 2015). Similarly, CSF lactate was elevated in patients with acute Lyme neuroborreliosis, but not in patients with neurosyphilis (Djukic et al., 2012). These data show that a shift of immune cell metabolism toward glycolysis occurs both in vitro and in vivo after an encounter with *Borrelia*. This indicates the involvement of a cellular glucose metabolism pathway in the pathogenesis of Lyme disease and possibly a target for drug development in long-term immune-mediated complications.

One of the strongest eQTLs was found for a SNP in *MGAT4C* involved in protein glycosylation (Mak et al., 2011), affecting *Borrelia*-induced IFN-γ responses. Glycosylation is known to be crucial for protein function, and recent studies identified defects in protein glycosylation causing profound immunodeficiency (Lyons et al., 2015). In addition, very recently it was reported that MGAT enzymes were linked to metabolic reprogramming (Ryczko et al., 2016), further supporting the role for metabolism in *Borrelia*-induced cytokine production. It is thus tempting to speculate that protein glycosylation might also be crucial for anti-*Borrelia* host defense mechanisms.

In conclusion, we used a functional genomics approach to describe the architecture of cytokine responses induced by *B. burgdorferi* in two large cohorts of healthy volunteers. We identified several crucial characteristics of *Borrelia* cytokine responses, such as a strong correlation with cytokine responses induced by intracellular bacteria, a strong influence of age on *Borrelia*-induced IL-22, and several genomic factors influencing these responses. Additionally, we demonstrate that a switch in glucose metabolism in immune cells is crucial for induction of cytokines by *Borrelia*. Future studies should aim to unravel the function of other cQTLs and genes identified here for the immune responses during Lyme disease.

EXPERIMENTAL PROCEDURES

Borrelia burgdorferi, Borrelia afzelii, and Borrelia garinii Cultures
Borrelia species were cultured at 25°C (and in a later time point at 34°C) as described in the Supplemental Experimental Procedures.

Escherichia coli
E. coli ATCC 25922 was grown overnight in culture medium, washed three times with PBS, and heat-killed for 60 min at 80°C. Aliquots were stored at −80°C throughout the study.

Staphylococcus aureus
S. aureus strain ATCC 29213 was grown overnight in culture medium, washed twice with cold PBS, and heat-killed for 30 min at 100°C. Aliquots were stored at −80°C throughout the study.

Mycobacterium tuberculosis
Cultures of H37Rv *M. tuberculosis* were grown to mid-log phase in Middlebrook 7H9 liquid medium (see Supplemental Experimental Procedures).

Borrelia Serology
Borrelia antibodies were measured using specific ELISAs (Serion/Virion Borrelia IgG; ESR-121-G and Borrelia IgM; ESR-121-M) and western blot analysis

(EuroImmun Borrelia IgG and IgM, DY-2131-3001-1G and DY-2131-3001-1M), both according to the manufacturer's instructions.

Ethics Statement

500FG and 200FG Cohorts

500FG is a cohort of 500 healthy individuals of Dutch European ancestry from the Human Functional Genomics Project (www.humanfunctionalgenomics. org). The cQTLs identified were validated in a second cohort of 200 healthy volunteers working as foresters in the Netherlands. Volunteers were asked to donate blood in order to determine the serum antibody response against *Borrelia*, since Lyme disease occurs as an occupational disease. None of the volunteers had an active *Borrelia* infection. In this cohort, all individuals gave written informed consent to donate an additional blood sample for research use. General characteristics of the cohorts have been described by ter Horst et al. (2016).

Isolation of Human PBMCs and Stimulation of Cytokine Production

Venous blood was drawn from the cubital vein of volunteers into 10 mL EDTA tubes (Monoject). Isolation of PBMCs was performed using Ficoll isolation, as described in the Supplemental Experimental Procedures. For measurements of metabolic parameters, see Supplemental Experimental Procedures.

Cytokine Measurements

Human IL-1β, TNF-α, IL-22, IL-17, IFN-γ, and IL-6 were determined by using commercial ELISA kits (PeliKine Compact, Sanquin, or R&D Systems) according to the manufacturer's instructions. The sensitivity of all assays was 20 pg/mL.

Genotyping, Quality Control, and Imputation

DNA samples of 500 individuals were genotyped using the commercially available SNP chip, Illumina HumanOmniExpressExome-8 v1.0. The genotype calling was performed using Opticall 0.7.0 (Shah et al., 2012) using the default settings. Samples with a call rate $\leq$ 0.99 (n = 18) were excluded from the dataset as were variants with an HWE $\leq$ 0.0001 and MAF $\leq$ 0.001. Potential ethnic outliers, identified by multi-dimensional scaling plots of our samples merged with 1000 Genome data (Figure S5), were also excluded (n = 17). This resulted in a dataset of 442 samples containing both cytokine and genotype information of 518,980 variants for further imputation. The strands and variant identifiers were aligned to the reference Genome of the Netherlands (Genome of the Netherlands Consortium, 2014) dataset using Genotype Harmonizer (Deelen et al., 2014). The data were phased using SHAPEIT2 v2.r644 (Delaneau et al., 2013) and imputed using IMPUTE2 (Howie et al., 2011) using the GoNL as the reference panel (Genome of the Netherlands Consortium, 2014). Post-imputation provided 7,512,899 variants. We selected 4,242,453 SNPs that showed MAF $\geq$ 5%, INFO score $\geq$ 0.8, and 3 samples per genotype for downstream cytokine QTL mapping.

Cytokine QTL Mapping

We used the 500FG dataset as a discovery cohort to identify genome-wide significant cQTLs since this cohort had the largest numbers of individuals (n = 489) in which both *Borrelia*-stimulated cytokine data and genome information was available. The 200FG dataset (n = 78) was used as validation cohort. See Supplemental Experimental Procedures.

RNA Sequencing, eQTL Expression, and Pathway Analysis

Candidate genes from significant cytokine QTL loci were further tested for responsiveness to any of the pathogens using RNA sequencing. See Supplemental Experimental Procedures for detailed information.

Microarray and qPCR

We obtained previously published transcriptome data for our analysis (GEO: GSE42606) (Smeekens et al., 2013). See Supplemental Experimental Procedures.

Cell Viability and Metabolite Measurements

Cell survival was analyzed in *Borrelia*- and medium-stimulated PBMCs using Annexin V/PI staining as described in the Supplemental Experimental Procedures. Lactate and glucose concentrations in cell culture supernatants were quantified using Amplex Red reagent (Thermo Fisher Scientific).

Extracellular Flux Measurements

Extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were measured using Seahorse XF^{e}96 analyzer (Seahorse Bioscience).

Western Blotting

Western blotting was performed using a TransTurboBlot system (Bio-Rad; Supplemental Experimental Procedures).

HIF-1α Knockout Experiments

Bone marrow and spleen cells from mice with an HIF-1α deficiency in the myeloid cell lineage were obtained from Dr. R.A. Cramer (Dartmouth, NH, USA). Spleen cells were stimulated with *B. burgdorferi* (10^6 and 10^7 spirochetes/mL, respectively), *Candida albicans* (C.a.), or anti-CD3/anti-CD28 antibodies for 6 days, after which supernatants were collected (Supplemental Experimental Procedures).

Transcriptome Analysis and Serum Lactate Measurements in Lyme Patients

RNA sequencing data of acute Lyme patients were obtained from a publicly available dataset (Bouquet et al., 2016) (GEO: GSE63085). Expression of selected genes was compared among EM patients at different time points and healthy controls by Kruskal-Wallis one-way ANOVA with Dunn's post hoc t test. Serum lactate concentrations were determined in a Romanian cohort of clinically proven EM patients and tick-bitten healthy controls. All patients were included before start of antibiotic treatment (conform IDSA guidelines), and samples were collected at baseline, after 2 weeks, and after 6 weeks.

Statistical Analysis

The data are expressed as mean ± SEM unless mentioned otherwise. Differences between experimental groups were tested using the Mann-Whitney U test performed on GraphPad Prism 4.0 software (GraphPad). p values of $\leq$ 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http:// dx.doi.org/10.1016/j.chom.2016.10.006.

AUTHOR CONTRIBUTIONS

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