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Identification of a Unique Hybrid Macrophage-Polarization State following Recovery from Lipopolysaccharide Tolerance

Christine O’Carroll,* Ailís Fagan,† Fergus Shanahan,* and Ruaidhrí J. Carmody‡

LPS tolerance is an essential immune-homeostatic response to repeated exposure to LPS that prevents excessive inflammatory responses. LPS tolerance induces a state of altered responsiveness in macrophages, resulting in repression of proinflammatory gene expression and increased expression of factors that mediate the resolution of inflammation. In this study, we analyzed the transcriptional plasticity of macrophages following LPS tolerance using genome-wide transcriptional profiling. We demonstrate that LPS tolerance is a transient state and that the expression of proinflammatory genes is restored to levels comparable to the acute response to LPS. However, following recovery from LPS tolerance a number of genes remained locked in a tolerizable state, including IL-33, CD86, IL-10, and NFIL3. Furthermore, we identified a number of genes uniquely induced following recovery from LPS tolerance. Thus, macrophages adopt a unique transcriptional profile following recovery from LPS tolerance and have a distinct expression pattern of regulators of Ag presentation, antiviral responses, and transcription factors. Our data suggest that recovery from LPS tolerance leads to a hybrid macrophage activation state that is proinflammatory and microbicidal in nature but that possesses a regulatory anti-inflammatory profile distinct from that of LPS-tolerant and LPS-activated macrophages.

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Inflammation is a powerful, organized, and complex immunological response to infection and tissue damage. TLRs are key sensors of microbial presence and host damage and are central to innate inflammatory responses (1). TLR4 is the most extensively studied TLR that recognizes LPS, an outer membrane cell wall component of Gram-negative bacteria. Ligation of TLR4 triggers intracellular-signaling pathways, culminating in the production of proinflammatory cytokines, chemokines, and type I IFNs to effectively clear infection (1–3). Chronic or repeated exposure of macrophage to LPS leads to a phenomenon termed LPS tolerance, which represents a state of altered responsiveness to additional LPS challenge. The most notable feature of LPS tolerance is the sharp reduction in proinflammatory cytokine expression, including TNF-α and IL-6, in response to LPS stimulation. LPS tolerance is critical in limiting the innate response to infection to reduce host damage and promote the resolution of inflammation (4–6). Failure to enforce LPS tolerance may lead to profound consequences, including death as the result of excessive and uncontrolled cytokine levels (4, 7).

Recent transcriptional profiling of macrophages identified two classes of LPS-responsive genes: those that are suppressed during LPS tolerance, so-called “tolerizable genes,” which include TNF-α and IL-6, and those genes whose expression is maintained or even increased during LPS tolerance, so-called “nontolerizable genes,” which include antimicrobial and anti-inflammatory genes. Chromatin modifications are associated with LPS tolerance, which promotes a global transcriptional switch in macrophage gene expression (8). This unique transcriptional signature was proposed to drive a phenotypic switch in macrophage polarization from a proinflammatory to an anti-inflammatory resolution state (8). A number of negative regulators of TLR-induced responses have been identified that are critical in promoting LPS tolerance. These include regulators of TLR4-induced signal transduction, such as IRAK-M, TOLLIP, SHIP, and A20, and transcriptional regulators, such as Bcl-3 (9–16).

Macrophages display a spectrum of activation states, which are broadly described as classical (M1), alternative (M2), and regulatory (17, 18). M1 macrophages are proinflammatory cytotoxic phagocytes and promote Th1 adaptive-immune responses following TLR and IFN-γ challenge (17, 18). In contrast, IL-4/IL-13 specifically polarizes macrophages to an alternatively activated M2 state. These cells are anti-inflammatory, promote tissue remodeling, and are important in mediating Th2 immunity to fungal and parasitic infections (17, 19, 20). Regulatory macrophages are immune regulatory cells that display both anti-inflammatory and proinflammatory features through suppression of some proinflammatory genes and increased production of IL-10, while maintaining Ag-presenting capacity. Immune complexes and FcR engagement in conjunction with TLR activation polarize macrophages toward a regulatory state. In addition, other stimuli, including glucocorticoids, PGs, and apoptotic cells, can polarize macrophages into a regulatory macrophage state (17). These three activation states represent the main groups in the emerging spectrum of macrophage polarization. However, because of the large number of activation stimuli that macrophages encounter in vivo,
many hybrid activation states exist. These include tumor-associated macrophages, intestinal macrophages, and LPS-tolerized (T) macrophages, all of which share overlapping features of M1, M2, and regulatory macrophages while displaying distinct markers of activation (21–26). LPS tolerance polarizes macrophages into an anti-inflammatory activation state distinct from M2 macrophage polarization (21–23). Although transcriptomics analysis has been performed on LPS tolerance (8, 22, 27), the plasticity of this state has not been explored using the same approach. In this study, we analyzed the persistence of LPS tolerance using transcriptional profiling and bioinformatics analysis. We used a model of LPS activation of murine bone marrow–derived macrophages (BMDMs) that generated naïve (N), LPS-activated/acute response (A = M1), and T polarization states. Our data demonstrate that LPS tolerance is a transient state in macrophages and that, following LPS tolerance, macrophages adopt a previously undescribed hybrid activation state, which we call “recovered macrophages” (RMs) to reflect the recovery from LPS tolerance. These data support previous in vivo and in vitro findings that LPS tolerance is transient (8, 27–29). Using genome-wide transcriptional analysis, we demonstrate that recovery from LPS tolerance, as defined by cytokine expression, is associated with a global change in the transcriptional profile of macrophages. Thus, RM cells express tolerizable genes, such as TNF-α and IL-6, following LPS stimulation at levels equivalent to LPS-activated M1-polarized cells. However, a number of genes remain locked in an LPS-tolerizable state and do not recover from LPS tolerance, distinguishing RM cells from M1 cells. Furthermore, RMs express a subset of inducible genes not induced in M1 or tolerized M2-like states. In addition, RM cells have a distinct expression pattern of regulators of Ag presentation and antiviral responses. Our data suggest that recovery from LPS tolerance leads to a novel hybrid macrophage activation state that is proinflammatory and microbicidal in nature but that possesses a regulatory anti-inflammatory profile distinct from that of T and LPS-activated (M1) macrophages.

Materials and Methods

Reagents

Ultrapure LPS from K12 Escherichia coli was used at a final concentration of 100 ng/ml (InvivoGen, Toulouse, France). Flow cytometry Abs (F4/80, CD80, CD86, CCR3, and isotype controls) were purchased from BD Biosciences (San Jose, CA). DMEM, FBS penicillin and streptomycin, t-glutamine, and nonessential amino acids were all purchased from Sigma-Aldrich (Dublin, Ireland).

LPS tolerance–recovery model

BMDMs were differentiated from bone marrow from isolated femur and tibia bones of 6–8-wk-old female C57BL/6 mice. BMDMs were differentiated in culture media supplemented with 30% L929-conditioned media for 7 d, as previously described (30). BMDMs were assessed by flow cytometry and were typically >95% F4/80+. BMDMs were cultured in complete BMDM media (DMEM, 10% FBS, 1% penicillin/streptomycin, 1% t-glutamine, and 1% nonessential amino acids). LPS tolerance was induced by stimulation with LPS (100 ng/ml) for 8 h, followed by washout of LPS with fresh medium. Cells were rested for an additional 16 h prior to restimulation with LPS (100 ng/ml) for 4 h. Recovery from LPS tolerance (R) was analyzed following an additional resting period of up to 4 d prior to restimulation with LPS (100 ng/ml) for 4 h to determine the optimal time point for recovery. All research, including transcriptional-profiling experiments, were performed on macrophages rested for 3 d prior to restimulation (R). Additional analysis of recovery from LPS tolerance on days 2 and 4 is specified within the text, where appropriate. The acute response to LPS was measured in cells that received no stimulation with LPS prior to stimulation with 100 ng/ml LPS for 4 h. Naive macrophages remained untreated. For microarray analysis, all groups, with the exception of the N group, were restimulated for 4 h prior to RNA isolation and subsequent downstream experiments (Fig. 1A).

Thiglycollate-elicted peritoneal macrophages

Peritoneal macrophages were isolated as previously described (31). Thiglycollate-elicted peritoneal macrophages were isolated from 6–8-wk-old female C57BL/6 mice by peritoneal lavage 3 d post-i.p. injection with 1 ml sterile 4% thiglycollate. Briefly, 10 ml ice-cold sterile PBS was injected into the peritoneal cavity, followed by gentle massaging of the cavity to allow cells to be collected and withdrawn by peritoneal lavage. Collected cells from three mice were pooled, passed through a 70-μm cell strainer (BD Biosciences), and centrifuged at 4°C for 5 min at 300 × g. Isolated thiglycollate-elicted peritoneal macrophage were allowed to adhere to tissue culture dishes overnight in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% t-glutamine, and 1% nonesential amino acids. Nonadherent cells were removed prior to experimental use.

Microarray profiling

All data processing was carried out using Bioconductor packages (32) in the R software environment. The data were background corrected using the Limma package (33). The data were then corrected for batch effects using the ComBat script (34). The corrected data were then normalized between arrays using quantile normalization (33). Differentially expressed genes were found using the Limma package (33). These genes were then corrected for multiple testing using the Benjamini–Hochberg method, with an adjusted p value cut off of p < 0.05 and a fold change of 1.5. Gene ontology (GO) was performed using DAVID (35). Gene-expression changes and gene-expression trends were visualized by hierarchical and K-means clustering using Genesis software (36). Gene-expression profiling was validated independently by quantitative real-time PCR (qRT-PCR). Microarray profiling data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database with the series entry identifier GSE47783.

Total RNA was isolated using the RNeasy mini kit (QIAGEN, Manchester, U.K.) with DNase treatment. RNA quantification and purity were measured using the Nanodrop ND1000 spectrophotometer (Thermo Scientific), with triplicate biological replicate samples submitted for microarray profiling (Beckman Coulter Genomics, Morrisville, NC). Briefly, 200 ng total RNA was fluorescently labeled with Cy3 nucleotides. Labeled RNA (cRNA) was hybridized to Agilent mouse 8 × 60K mouse microarrays (Agilent-028005). Hybridized arrays were washed and scanned, with data extrapolated for downstream bioinformatics analysis. Each experiment is representative of BMDM derived from bone marrow pooled from three mice. Microarray gene-expression profiling was performed in triplicate.

Gene-expression analysis

Gene expression was measured by qRT-PCR. A total of 1 μg total RNA was used to synthesize cDNA with random hexamer primers using Transcriptor Reverse Transcriptase (Roche Applied Sciences, West Sussex, U.K.). The reaction mix was incubated for 10 min at 25°C to allow efficient annealing of random primers, followed by incubation at 55°C for 30 min. The enzyme was inactivated at 85°C for 5 min. Gene expression was measured by qRT-PCR using the LightCycler 480 (Roche Applied Sciences). Primers were designed using the Universal Probe Library system (Roche Applied Sciences). Gene expression for individual genes was measured and normalized to 18S (forward = 5′-AGAACGATTTGCTGTTCACTGTC-3′, reverse = 5′-GCTTCTAATCCACGACATTCCAA-3′). Gene-expression changes were calculated using the 2−DDCT method (37). Data are represented as relative mRNA. All research was performed under nuclease-free conditions.

Western blotting

BMDMs were removed from tissue culture dishes using a cell scraper, with cells collected into 1× ice-cold PBS (30). BMDMs were washed twice in ice-cold PBS and centrifuged at 300 × g for 5 min at 4°C. Total protein was isolated by lysing the cell pellet in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 μM Na3VO4, 1 mM EDTA) supplemented with a protease and phosphatase inhibitor mixture (Sigma-Aldrich). Cytoplasmic and nuclear proteins were isolated from BMDMs by differential centrifugation. Protein quantification was performed using a BSA standard curve and Bradford reagent. Protein concentration was measured at 595 nm using an absorbance plate reader. Total protein was resolved by SDS-PAGE gels, transferred to polyvinylidene difluoride membrane, and immune blotted for p65, c-Rel, p50, RelB, IRF4 (Santa Cruz, Dallas, TX), IRF5 (Abcam, Cambridge, U.K.), p-p38, p38, p-ERK, and ERK (Cell Signaling, Boston, MA).
Flow cytometry

Macrophages were removed from tissue culture dishes and collected in ice-cold 1× staining buffer (1× PBS, 0.5% BSA, 1% FBS, 0.1% sodium azide [Sigma-Aldrich]) and washed three times in ice-cold staining buffer. Cells were incubated with CD16/CD32 Ab for 15 min, according to the manufacturer’s protocol, to block nonspecific binding of Abs to the FcRs on cells. Cells were surface stained with Abs to F4/80, CD80, CD86, and CCR3 in the dark on ice for 30 min. The cells were washed in staining buffer and resuspended in running buffer (1× PBS, 1 mM EDTA, 0.5% BSA) prior to analyzing on the Accuri C6 Flow cytometer (BD Bioscience). Isotype controls and fluorescence minus one (FMO) were used as controls. Data were analyzed using FCS express flow cytometry software (De Novo Software, Los Angeles, CA).

Cell viability

Viability of BMDMs was measured by quantifying ATP levels from cells of each group (N, A, T, and R) using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) at the experimental end point. Briefly, cells were allowed to reach room temperature, then an equal volume of CellTiter-Glo reagent was added and the mixture was shaken for 10 min. ATP levels were measured using an ATP standard curve, with luminescence recorded using a luminometer. Data are represented as the percentage viability.

Statistical analysis

Differences between groups were determined using one-way ANOVA, with Tukey post hoc analysis. qRT-PCR expression data were calculated using the 2−ΔΔCT methods (37) and are expressed as relative mRNA. Statistical analysis was performed using GraphPad Prism software (San Diego, CA). Data are mean ± SEM and a p value < 0.05 was considered statistically significant.

Results

Recovery from LPS tolerance involves a global shift in the transcriptional profile

We designed a model of LPS tolerance in ex vivo BMDMs based on previous studies (8, 22, 27) of tolerance in human monocytes and mouse BMDMs. We generated a transcriptional profile of N, M1 (A), and T macrophages. We also analyzed LPS responses in RM cells (Fig. 1A). Briefly, LPS tolerance was induced by treatment with LPS (100 ng/ml) for 8 h, followed by washout of LPS with fresh medium. Cells were rested for an additional 16 h prior to restimulation with LPS (100 ng/ml) for 4 h. Recovery from LPS tolerance was analyzed following an additional resting period of 3 d prior to restimulation with LPS (100 ng/ml) for 4 h. The acute response to LPS was measured in cells that were not stimulated with LPS prior to stimulation with 100 ng/ml LPS for 4 h. N macrophages remained untreated (Fig. 1A).

The induction of LPS tolerance under the conditions used in this study was confirmed by qRT-PCR of three previously described LPS-tolerizable proinflammatory genes: IL-6, CXCL10, and TNF-α (8) (Fig. 1B). LPS-inducible expression of these genes was suppressed in the T group compared with the A group. In contrast, cells in the RM group demonstrated inducible gene expression in all three genes at levels comparable to the A group. Similar expression patterns of IL-6, CXCL10, and TNF-α were seen in thioglycollate-elicited peritoneal macrophages treated in the same ways BMDMs (Fig. 1C). The viability of cells recovering from tolerance was similar to that of tolerized cells (Fig. 1D). This confirms that LPS tolerance is a transient macrophage-activation state in both mouse and human macrophage/monocyte cells (Fig. 1B, 1C), in agreement with previous in vitro and in vivo studies (8, 27–29, 38).

We next performed microarray analysis of LPS-stimulated BMDMs in each of the groups described above. Initial K-means clustering analysis revealed a unique transcriptional signature for each macrophage treatment group (N, A, T, and RM), with 10 unique clusters that grouped genes with similar expression profiles across each macrophage group (Fig. 2A). Cluster 1 contains genes that are highly induced by LPS in the T group, but not the A or RM group, illustrating that clusters of genes are specific to the induction and maintenance of LPS tolerance in macrophages. In contrast, clusters 3 and 5 contain genes that were suppressed in T group but were inducible again in the RM group. Interestingly, cluster 5 genes were induced to a greater level in the RM group relative to the A group, whereas cluster 3 genes were induced to a lesser degree. This suggests that the transcriptional profile of RMs is different from that of A group. In support of this finding, cluster 6 contains genes that are highly induced in the RM group compared with all other groups, thus identifying a unique transcriptional signature for this group (Fig. 2A).

We next performed correspondence analysis (COA) on the microarray dataset to visualize and measure the correlation between the global transcriptional profile of each group (N, A, T, and RM). A 30% principal component 1 (PC1) measure of variance between the different macrophage groups was identified, with the A and RM populations clustered closely together. This demonstrated that the A and RM groups share overlapping transcriptional profiles distinct from N and T groups.

FIGURE 1. Recovery from LPS tolerance restored macrophage proinflammatory gene expression. (A) Ex vivo model of recovery of LPS tolerance using murine BMDMs, as described in Materials and Methods. Four unique macrophage-polarization states were induced: N, A, T, and RM (day 3). (B) CXCL10, IL-6, and TNF-α gene expression was measured by qRT-PCR for each macrophage-activation state 4 h postrestimulation (BMDMs). (C) Gene expression of CXCL10, IL-6, and TNF-α was measured in thioglycollate-elicited peritoneal macrophages. (D) BMDM viability (% viability) was measured 4 h post-LPS restimulation. Data are representative of more than four independent experiments and are shown as relative mRNA. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey post hoc test. D0, Day 0; D-1, day 1; D-2, day 2; D-3, day 3.
from those of the N and T groups. Upon closer examination, PC4 (8% variance between groups) identified transcriptional differences between the A and R groups, further supporting a unique gene-expression profile in RM cells (Fig. 2B). This finding indicates a high degree of macrophage plasticity following the induction of LPS tolerance. Taken together, these initial findings confirm that LPS tolerance is a transient macrophage state and that recovery from LPS tolerance involves a global change in the macrophage transcriptional profile. Each macrophage group in our model represents a distinct macrophage state; however, the gene-expression profile of the RM group shares some similarities with that of the A group. This suggests that recovery from tolerance polarizes macrophages toward a proinflammatory classically activated–like (M1) state.

Differentially expressed genes and GO enrichment

To further characterize the gene-expression signature of RM cells, we identified differentially expressed genes in the RM group relative to the N, A, and T groups (±1.5-fold change, p < 0.05). GO analysis was used to determine the biological significance of differentially expressed genes. Unsurprisingly, “immune response,” “TLR signaling,” “Ag presentation,” “cytokine regulation,” and “defense response” were the most significantly enriched biological processes found from the analysis of genes upregulated in the RM group relative to the T group (Supplemental Fig. 1B, Supplemental Tables I, II). These data suggest that, following recovery from LPS tolerance, macrophages are proinflammatory cells with increased cellular proliferative capacity compared with anti-inflammatory tolerated cells.

Although COA (PC1: 30%) revealed similar transcriptional profiles between the A group and the RM group (Fig. 2B), clear differences in the expression of distinct groups of genes were found. Following recovery from tolerance, restimulation of macrophages with LPS induced the expression of 310 genes not induced during the initial acute LPS response. Conversely, 332 genes were no longer induced by LPS following recovery from tolerance. This finding is supported by COA (PC4: 8%) (Fig. 2B). The chemokine receptor CX3CR1, which is often used to distinguish between inflammatory and resident monocyte/macrophage lineages (25, 39–42), was one of the most significantly induced genes following recovery from LPS tolerance but not during the acute LPS response. Similarly, the chemokine receptors CCR5 and CCR3, the folate receptor FOLR2, and IFN-γ were specifically induced to high levels in the R group (Figs. 3A, 4, Supplemental Table I). The proinflammatory gene IL-33 was the most significantly suppressed differentially expressed gene in the R group relative to the A group. IL-33 is suppressed during LPS tolerance and remains “locked” in a tolerizable gene-expression state following recovery from LPS tolerance (Figs. 3B, 4B, Supplemental Table I).

Unsurprisingly, we found that the 310 LPS-induced genes differentially expressed in the RM group relative to the A group were enriched with numerous immune-related processes and pathways by GO analysis. These included “immune response,” “response to wounding,” and “inflammatory response” (Fig. 3C, Supplemental Tables I, II). Of note, the most significant biological terms identified from the downregulated differentially expressed genes were “antiviral response” and “response to virus.” Genes identified in these groups include IFNA4, IFNA12, IFNA13, IFNA2, IFNA5, and IFNA7 (Fig. 4A, 4B). Other significantly enriched GO terms were “cytokine activity,” “immune system development,” and “cytosolic DNA-sensing pathways” (Fig. 3C, Supplemental Tables I, II). Genes associated with these terms include the antinflammatory cytokine IL-10 and transcription factor NFKB1 (Fig. 4A, 4B, 4D). Taken together, the transcriptional profile of RM cells reflects many features of A cells; however, these cells have a unique transcriptional signature that identifies them as a novel macrophage-activation state, with altered expression of regulators of the antiviral responses.

Recovery from LPS tolerance induces a hybrid macrophage-activation state

Ag presentation is a classical feature of M1 macrophages, whereas M2 anti-inflammatory macrophages display a reduced capacity to present Ags to adaptive immune cells (17, 43). We identified a significant increase in the surface expression of CD86 and CD80 cells following acute activation with LPS (Fig. 5A). CD86 surface expression was suppressed during LPS tolerance and was not further inducible following recovery from LPS tolerance. Similarly, LPS-inducible CD80 surface expression was suppressed in the T group and was not inducible in the RM group (Fig. 5A). In addition, LPS-inducible CD86 mRNA levels were suppressed in both the T and RM groups, similar to IL-33, IL-10, NFKB1, and type 1 IFN.
genes (Figs. 4A, 4B, 4D, 5B). Interestingly, we measured no difference in the LPS-inducible levels of NOS2 mRNA between cells in the RM and A groups, as determined by both microarray and qRT-PCR gene-expression analysis (Fig. 5C).

To further define this inducible macrophage hybrid RM state, we analyzed M1- and M2-polarizing markers from the microarray dataset. Using hierarchical clustering analysis, we identified an overlap in M1- and M2-associated genes expressed following recovery from tolerance (Fig. 5D). The M1-polarization markers IL-6, NOS2, and IL-12B (17, 18, 43) were significantly induced by LPS following recovery from tolerance, similar to the acute-LPS response. A number of M2-associated polarization markers, such as CCL17 and CCL24 (26), were also significantly induced following recovery from tolerance. Furthermore, there was an LPS-induced increase in the expression of the M2-polarization markers MRC1 and chi3l3 (17, 26) following recovery from tolerance (Fig. 5D). These data suggest that recovery from LPS tolerance may alter macrophage Ag-presentation capacity and subsequent activation of the adaptive-immune response. Our finding that, following recovery from tolerance, LPS may robustly induce iNOS gene expression suggests that these cells retain their cytotoxic microbicidal function. In addition, the expression of both M1- and M2-associated genes in LPS-treated cells following recovery from LPS tolerance induces a hybrid macrophage state with a unique transcriptional signature.

Recovery from LPS tolerance is associated with a unique signaling and transcription factor profile in macrophages

Macrophage polarization is regulated by a number of key transcription factors, including the NF-κB and IRF families. To assess the role of NF-κB in shaping macrophage responses following recovery from LPS tolerance, we initially assessed the expression of NF-κB subunits by immunoblot. The levels of RelB, c-Rel, and p65 subunits in the RM group were similar to those found in the A group (Fig. 6A). In contrast, the T group demonstrated elevated levels of RelB, c-Rel, and p50 protein (Fig. 6A). Interestingly, total p50 levels remained elevated in the RM group. However, in contrast to tolerized cells (14), increased p50 protein is not found in the nucleus prior to restimulation, indicating that elevated p50 protein is not repressive in RMs (Fig. 6B). In addition, the
restimulation with LPS was important for translocation of NF-κB into the nucleus in all groups (Fig. 6B). We next measured IRF4 and IRF5 protein levels, which were associated with macrophage polarization (44, 45). IRF4 protein levels were reduced following recovery from tolerance, whereas no differences in IRF5 protein levels were observed (Fig. 6C). To assess LPS-induced signal transduction following recovery from LPS tolerance, we used phospho-specific Abs to measure activation of the ERK1/2 and p38 kinases following LPS stimulation for 15, 30, or 60 min (Fig. 6D). p38 and ERK phosphorylation was suppressed in the T group at 30 min, in agreement with previous studies (46). Following recovery from tolerance, p38 phosphorylation was increased relative to the acute LPS response. ERK1/2 were activated to levels comparable to those of the acute LPS–activated cells (Fig. 6D).

Hierarchical clustering analysis of transcription factor mRNA levels revealed no difference in the expression of NF-κB and STAT mRNA in the RM group compared with the A group. However, we observed differences in the levels of the transcription factors NFIL3 and IRF8, a member of the IRF family of transcription factors. LPS-inducible NFIL3 and IRF8 gene expression was suppressed following recovery from tolerance in a pattern similar to IL-33, IL-10, and the type 1 IFN genes (Fig. 6E). These data suggest that, although no differences were observed in NF-κB protein levels, suppression of IRF4, IRF8, and NFIL3 may play a role in defining the transcriptional signature following recovery from LPS tolerance.

**Discussion**

The acute response to LPS or endotoxin is one of the most extensively studied immune processes, because of its powerful effects on host immunity and disease. In this study, we used a transcriptomics approach to characterize the transcriptional signature of macrophages post-LPS tolerance induction. We identified that recovery from LPS tolerance induced a macrophage-activation state involving a global transcriptional shift from an anti-inflammatory profile to a hybrid macrophage state. Although a large proportion of tolerizable proinflammatory gene expression (IL-6, TNF-α, CXCL10) was restored in RM cells, a number of proinflammatory (IL-33, type 1 IFNs), anti-inflammatory (IL-10), and immune-regulatory genes (NFIL3, CD86) remained locked in a tolerizable state, and, thus, failed to recover from LPS tolerance. The transcriptional profiles generated appear to reflect the individual cell rather than a heterogeneous population, because flow cytometry analysis of cell surface markers did not reveal subpopulations of cells. In addition, RMs expressed a number of unique markers not expressed in the other macrophage groups. Taken together, these data confirm that recovery from LPS tolerance does not polarize...
Macrophages express reduced levels of two associated M2-polarizing markers (IL-33, IRF4), which may be critical to promoting this hybrid state. The reduced levels of IRF4 protein observed are not reflected in the mRNA levels, suggesting that IRF4 may be posttranslationally regulated in RMs. IL-33 is a proinflammatory cytokine, alarmin, and amplifier of LPS activation (50–54). The lack of IL-33 expression in RMs may be important in limiting the amplification of LPS responses and M2 polarization through paracrine signaling. Reduced expression of the M2-polarizing transcription factor IRF4 (44, 55) may play a role in facilitating recovery from LPS tolerance by limiting the potential for maintenance of an M2 macrophage-polarization state. IRF4 was shown to be important for the induction of M2-associated genes in response to chitin in vivo (44) and in vitro (45). Previous studies (45, 56) demonstrated that macrophage polarization may be skewed toward an M2 profile following differentiation with M-CSF but not GM-CSF, suggesting that differentiating factors may influence the role of specific transcription factors in promoting activation states. Some M2-associated genes, including CCL17 and CCL22, also may be induced in response to TLR activation (8, 27). Thus, M2-associated genes that are LPS responsive may influence the role of specific transcription factors in promoting activation states.

We found increased expression of CX3CR1 in the RM and T groups. CX3CR1 is expressed on some resident macrophage populations and has been implicated in cellular survival. Thus, increased expression of CX3CR1 in RMs may regulate survival postinflammatory challenge (57, 58). Although the reduced expression of a number of type 1 IFNs in RMs may increase susceptibility to viral infections, suppression of these genes may be important to limit their unnecessary production postbacterial inflammatory challenge. Our data demonstrate that RMs are distinct from LPS-tolerized macrophages and represent a unique macrophage population primed to respond to new inflammatory challenges under strict environmental conditions.

The plasticity of macrophages could potentially have both positive and negative outcomes, depending on the local environ-
mental requirements and activation stimuli that macrophages encounter. Although an endotoxin/LPS-tolerant state may limit potentially devastating consequences of excessive inflammation, prolonged tolerance can have devastating consequences. Monocytes isolated from patients with sepsis or cystic fibrosis display reduced responsiveness to ex vivo challenges with LPS, as determined by proinflammatory cytokine production (59). These cells remained locked in a tolerant state that coincided with patient mortality due to an inability to produce a robust inflammatory response to secondary infections. Interestingly, patients who recovered from sepsis are responsive to LPS upon LPS challenge ex vivo (59, 60). This suggests that recovery from endotoxin tolerance may be a critical physiological response to maintain host protection to invading microbial challenges. In addition, the high levels of IFN-γ expressed in LPS-stimulated RMs, but not in tolerized cells or during the acute response, may function to elicit a heightened state of immune readiness that prepares the host for additional or repeated infections.

Under normal homeostatic conditions, intestinal macrophages adopt an anti-inflammatory tolerant-like state toward the local environmental microbial community. This environment is rich in the polarizing cytokines IL-10 and TGF-β, which contribute to an anti-inflammatory–tolerant environment in the gut (25). In addition, the resident Kupffer cell population in the liver adopts an anti-inflammatory tolerized-like phenotype specific to the liver microenvironment, where they are exposed to a wide array of gut-derived and environmental toxins under normal physiologic conditions (48, 61, 62). These two macrophage populations represent environments in which prolonged tolerance is a physiological protection mechanism. The loss of this normal tolerance state is associated with alcoholic liver disease, hepatitis C viral infections, and inflammatory bowel disease (63, 64). Of note, deficiencies in IL-10 and the transcription factor NFIL3, two genes that are locked in a tolerized state in RMs, have been associated with chronic inflammation in the gut (65). In addition, CD14⁺ lamina propria mononuclear cells from patients with Crohn’s disease or ulcerative colitis showed reduced NFIL3 gene expression in comparison with noninflamed cells (65). NFIL3 is a regulator of IL-12B expression and is induced by IL-10 in macrophages. Although we
measured no difference in LPS-inducible IL-12B gene expression in RMs, the suppression of both IL-10 and NFIL3 expression in RMs may facilitate the recovery from LPS tolerance. Thus, these data suggest that recovery from LPS tolerance may be central to restoring normal physiological responses following microbial infections. Importantly, recovery from a tolerant state may be environment specific, as a result of the positive and negative effects that a loss of tolerance may have on host immunity.

In summary, we identified a novel transcriptional signature in macrophages following recovery from LPS tolerance that has both proinflammatory and anti-inflammatory characteristics. Recovery from LPS tolerance may represent a physiological response of macrophages following microbial infection that is important in controlling and limiting excessive inflammation. Macrophages that are in an LPS-tolerance state may be more susceptible to new microbial challenges as a result of reduced inducible NO synthase activity and Ag-presentation capability. Macrophages remaining locked in this tolerized state likely would have a significant impact on the host response to new microbial challenges. Therefore, recovery from LPS tolerance may prime macrophages to mount an effective immune response against new microbial challenges while maintaining a level of tolerance that protects against unnecessary overactivation of the inflammatory response.

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Disclosures

The authors have no financial conflicts of interest.

References