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TLR7 and TLR8 Differentially Activate the IRF and NF- κ B Pathways in Specific Cell Types to Promote Inflammation

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ABSTRACT

TLR7 and TLR8 are pattern recognition receptors that reside in the endosome and are activated by ssRNA molecules. TLR7 and TLR8 are normally part of the antiviral defense response, but they have also been implicated as drivers of autoimmune diseases such as lupus. The receptors have slightly different ligand-binding specificities and cellular expression patterns that suggest they have nonredundant specialized roles. How the roles of TLR7 and TLR8 differ may be determined by which cell types express each TLR and how the cells respond to activation of each receptor. To provide a better understanding of the effects of TLR7/8 activation, we have characterized changes induced by TLR-specific agonists in different human immune cell types and defined which responses are a direct consequence of TLR7 or TLR8 activation and which are secondary responses driven by type I IFN or cytokines produced subsequent to the primary response. Using cell sorting, gene expression analysis, and intracellular cytokine staining, we have found that the IFN regulatory factor (IRF) and NF- κ B pathways are differentially activated downstream of the TLRs in various cell types. Studies with an anti-IFNAR Ab in human cells and lupus mice showed that inhibiting IFN activity can block secondary IFN-induced gene expression changes downstream of TLR7/8 activation, but not NF- κ B-regulated genes induced directly by TLR7/8 activation at earlier timepoints. In summary, these results elucidate the different roles TLR7 and TLR8 play in immunity and inform strategies for potential treatment of autoimmune diseases driven by TLR7/8 activation. *ImmunoHorizons*, 2020, 4: 93–107.

INTRODUCTION

TLR7 and TLR8 are pattern recognition receptors that normally function to recognize ssRNA molecules originating from viruses. Although some characterization of their ligand recognition specificities has been performed (1–3), there is still only minimal knowledge about the endogenously occurring molecules that activate these receptors. This is notable, as responses driven by activation of these receptors may be different if the stimulus is a synthetic small molecule or larger RNA species (1, 4, 5). Although TLR7 and TLR8 likely evolved to be sensors of foreign viral RNA, they may also respond to endogenous RNA molecules and drive

Type I IFN production, inflammation, and autoimmunity associated with diseases such as systemic lupus erythematosus (SLE) in which autoantibodies and immune complexes containing RNA molecules are generated (6). It is also possible that microRNA or other RNA molecules may stimulate these TLRs to drive autoimmunity (7–10).

Understanding which cell types express TLR7 and TLR8 and defining exactly how the cell types uniquely respond to activation of the two receptors would greatly aid in characterizing how the TLRs may contribute to autoimmunity. Currently, it is generally thought that B cells, monocytes, and plasmacytoid dendritic cells (pDCs) are the primary cell types expressing TLR7, but the cell

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Abbreviations used in this article: DC, dendritic cell; IFNAR, IFN- α/β receptor; IFN Hi, IFN high; IFN Lo, IFN low; IRB, institutional review board; IRF, IFN regulatory factor; KO, knockout; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; SLE, systemic lupus erythematosus.

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types expressing TLR8 have been less well defined, although earlier work suggested myeloid dendritic cells (mDCs) and monocytes are the most TLR8 responsive (11). Recombinant expression of the two TLRs is difficult, and thus generation of quality Abs for Western blot detection has not been very successful. Some studies have evaluated TLR7/8 mRNA expression as a means for defining cell type localization, but such studies would be better supported by detection of TLR7/8 protein or activity.

Evidence exists suggesting that TLR7 and TLR8 may drive SLE or other autoimmune diseases, but it is not known which of the TLRs plays a larger role in disease pathogenesis. Single nucleotide polymorphisms in the TLR7/8 pathways (12), as well as other genetic data, and some mouse model data suggest that these TLRs may contribute to SLE development by driving production of type I IFN that promotes breaks in tolerance and disease (13). Additional evidence implicating these TLRs is the fact that hydroxychloroquine (Plaquenil), which may inhibit endosomal TLR signaling, is often used as a standard of care for SLE (14). However, investigating the role of these TLRs in autoimmunity and disease in greater detail is challenging because of the lack of available selective inhibitors and unclear TLR8 activity in mice. Although TLR7 (15) and TLR8 (16) overexpression in mice is sufficient to drive autoimmune disease, the role of TLR8 in driving disease under normal circumstances is less obvious, and sequence differences between mouse and human TLR8 makes the comparison even more difficult. TLR7 has been studied in the context of lupus in the BXS^B-Yaa mouse model (15), which involves duplication of the *TLR7* gene in males and also the pristane-induced lupus mouse model in which *TLR7* knockout (KO) can prevent disease (17, 18). Given the currently available evidence, hypothetically, TLR7 and/or TLR8 may drive disease pathogenesis in a subset of SLE patients. Indeed, both *TLR7* and *TLR8* are on the X chromosome, consistent with the observation of higher levels of expression in females (19, 20); thus, more TLR7/8 activity in females may be one reason for the higher prevalence of SLE in females. Additionally, studies have suggested a link between IFN and autoantibodies (21–23), and it has been hypothesized that RNA-binding autoantibodies can activate TLR7/8 to drive IFN and inflammation that contributes to or even drives SLE disease activity (23).

Although TLR7 and TLR8 are both located endosomally and both respond to RNA ligands, their signaling pathways may differ depending on the cell type and the activating ligand. Furthermore, the downstream responses to these two TLRs may be different, depending on cell type. Evidence has suggested that the IFN regulatory factor (IRF) and NF- κ B signaling pathways are the primary pathways activated downstream of TLR7/8. Activation of these pathways culminates in the production of type I IFN or inflammatory cytokines, and the cellular regulation of these pathways is complex and not fully understood (4, 24). There may be some cell type-specific differences in the signaling molecules downstream of TLR7/8 and, consequently, the responses they produce upon activation. In addition, cross-talk between endosomal TLRs, especially between TLR7 and TLR8 as well as between TLR7 and TLR9, may influence subsequent activation

of different pathways (25). If cross-talk alters the activity of specific TLRs, it may be affected by the TLRs that are coexpressed in a cell. Understanding in more detail the signaling pathways and responses downstream of TLR7 and TLR8 would help to define which TLR drives different aspects of an immune response and inflammation in the case of autoimmune disease.

In the work reported in this paper, we have found that TLR7 and TLR8 have distinct cell expression patterns, and the downstream signaling of these TLRs is not identical, as they have different IRF and NF- κ B pathway biases and serve nonredundant functions. The findings also provide insight into how these TLRs may contribute to autoimmunity and how their inhibition may provide potential benefits for the treatment of autoimmune disease beyond what is provided by inhibition of the IFN pathway alone.

MATERIALS AND METHODS

Materials

Treatment of whole blood with R848 with or without anifrolumab. Whole blood was obtained from healthy adult volunteers in an institutional review board (IRB)-approved procedure. Subjects had freely consented to participation in the study, and sampling was conducted using safe protocols approved by the Western IRB. Briefly, blood was collected into sodium heparin tubes and stimulated with either 1 μ M R848 (InvivoGen), 100 U/ml of IFN- α , or DMSO as a control for 1, 2, 4, 6, or 24 h at 37°C, 5% CO₂. There were additional 24 h timepoint treatments with R848 or DMSO in the presence or absence of 1 μ g/ml of the anti-IFNAR Ab anifrolumab (Creative Biolabs). After incubation, the samples were transferred to PAXgene RNA Tubes (QIAGEN) and frozen for later gene expression analysis.

FACS sorting of stimulated cells. Whole blood was collected in sodium heparin tubes and treated with 5 μ M of the TLR7-specific agonist CL-087, 2 μ M of the TLR8-specific agonist motolimod (Selleck Chemicals), or DMSO for 1 h or 24 h at 37°C 5% CO₂. After incubation, RBCs were lysed with 1 \times Pharm Lyse (BD Biosciences) for 20 min at 4°C. All subsequent steps were performed on ice unless otherwise indicated. The cells were spun down at 500 \times g for 5 min and washed with PBS twice. For detection of T cells, B cells, monocytes, granulocytes, and dendritic cells (DCs); cells were stained with surface markers anti-CD3 FITC (clone UCHT), anti-CD14 BV510 (clone M ϕ P9), anti-CD19 PE (clone HIB19), and anti-HLA-DR allophycocyanin/cyanine 7 (clone G46-6) (BD Pharmingen and BioLegend) for 30 min at 4°C in the dark, followed by two washes with FACS buffer (PBS with 5% FBS). The stained cells were FACS sorted to obtain 50,000 DCs, 100,000 monocytes, and 200,000 B cells, T cells, and granulocytes using the FACSaria. Cells were sorted directly into 350 μ l of RNeasy lysis buffer (RLT) (QIAGEN). RNA extraction was performed using the RNeasy Micro Kit (QIAGEN) and the QIAcube.

PBMC isolation and intracellular cytokine analysis by flow cytometry. Whole blood was layered in 50-ml ACCUSPIN Tubes

containing Histopaque (Sigma-Aldrich). PBMCs were isolated by collecting the PBMC monolayer after 20 min centrifugation at $1000 \times g$. The collected PBMCs were washed with PBS and resuspended to 2×10^6 cell/ml in RPMI 1640 containing 10% FBS and penicillin/streptomycin. PBMCs were stimulated at 2×10^6 cell/ml with either 1 μ M R848, 5 μ M CL-087, or 2 μ M motolimod in the presence of 1 μ l/ml GolgiPlug (BD Biosciences) for 4 or 24 h at 37°C 5% CO₂. After incubation, the PBMCs were spun down at $500 \times g$ for 5 min, washed twice with PBS, and resuspended in PBS. Cells were stained using 1 μ l/ml of viability dye (BD Biosciences) for 15 min at room temperature in the dark. Following the incubation, cells were washed in FACS buffer (PBS with 5% FBS) and stained with surface markers for CD3 allophycocyanin-H7 (clone SK7), CD14 PE-cyanine 7 (clone M5E2), CD19 BUV496 (SJ25C1), CD1c BV786 (F-10/21A3), HLA-DR allophycocyanin-R700 (G46-6), CD16 BV711 (3G8), CD123 BV510 (9F5), CD27 BB700 (M-T271), IgD PE-CF594 (IA6-2), and CD303 BUV395 (V24-785) in the presence of human Fc block (Invitrogen) for 20 min at 4°C in the dark. After incubation, cells were washed in FACS buffer then incubated with fixation/permeabilization buffer (BD Biosciences) for 20 min at 4°C in the dark. Following this treatment, cells were washed twice with Perm/Wash Buffer (BD Biosciences) and stained intracellularly for IFN- α PE (7N4-1), IL-6 BV421 (MQ2-13A5), IL-1 β AF647 (H1B-98), and TNF- α BV650 (MAb11) for 30 min at 4°C in the dark. Cells were then washed twice with Perm/Wash Buffer and resuspended in FACS buffer before analysis using the BD LSRFortessa $\times 20$. Data processing was performed using FlowJo software and Cytobank. For this analysis, DCs were subsetted into mDC and pDC populations (see Supplemental Fig. 1 for full gating strategy) according to known markers.

Pristane mouse studies. All procedures using animals were performed in accordance with the EMD Serono Institutional Animal Care and Use Committee and all local and national laws and regulations regarding animal care. The pristane lupus mouse model was run as described previously (26). Briefly, female DBA/1 mice used for pristane model studies were purchased from The Jackson Laboratory. To induce disease development, DBA/1 mice were injected with 0.5 ml of pristane (Sigma-Aldrich) i.p. at 11–12 wk of age. Beginning at 2 mo after pristane injection, mice were treated with the anti-IFN- α/β receptor (IFNAR) Ab MARI-5A3. At 1, 4, or 8 wk after treatment, mice were euthanized via CO₂ asphyxiation, and blood was collected via the vena cava and stored in RNeasy Protect Animal Blood Tubes (QIAGEN) and frozen to -80°C for later analysis.

NanoString analysis. NanoString was chosen as the platform for gene expression analysis, as it is a robust and simple technology that avoids amplification bias associated with other gene expression technologies. The platform is easily customizable for a gene list of interest and allows for multiplex detection of more targets than conventional PCR, but data analysis is simpler than for RNA next generation sequencing. These features made NanoString the best choice for these studies, as our goal was to measure

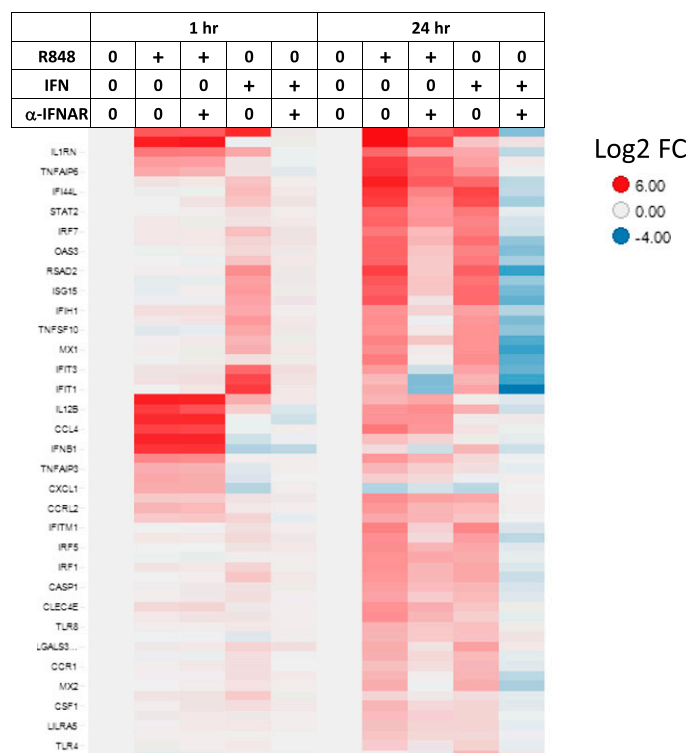


FIGURE 1. Identification of genes induced in whole blood directly by TLR7/8 activation or secondarily by IFN.

Human whole blood was pretreated in the absence or presence of anifrolumab (1 μ g/ml) for 15 min, followed by treatment for 1 or 24 h with R848 (1 μ M), IFN- α (100 U/ml), or DMSO at 37°C. After incubation, the blood was added to PAXgene RNA Tubes for preservation, and RNA was subsequently extracted and analyzed for gene expression by NanoString. The heat map shows the log₂ fold change compared with the DMSO group at each timepoint. Only genes with a log₂ fold change >1 are presented. Data are the average of two donors and representative of two separate experiments.

expression of a number of select genes in the pathways downstream of TLR7 and TLR8 activation in the most practical and robust manner possible. RNA was isolated from blood samples stored in PAXgene RNA Tubes using the PAXgene Blood RNA Kit (QIAGEN). The RNeasy Protect Animal Blood Kit was used for isolation of RNA from mouse blood samples, and the RNeasy Micro Kit was used for RNA purification from sorted cells. All QIAGEN RNA isolation kits were run on the QIACube robot. A custom 93-gene panel was designed for NanoString analysis that included six housekeeping genes for normalization. Both human and mouse versions of the panel were produced and used for analysis of each species. Purified RNA samples were hybridized overnight to gene-specific reporter probes at 65°C and analyzed using the NanoString Prep Station and nCounter instruments according to the manufacturer instructions. The NanoString Human Immunology v2.0 panel was used for confirmational analysis of sorted cell population's phenotype. NanoString data analysis was performed using the nSolver software program.

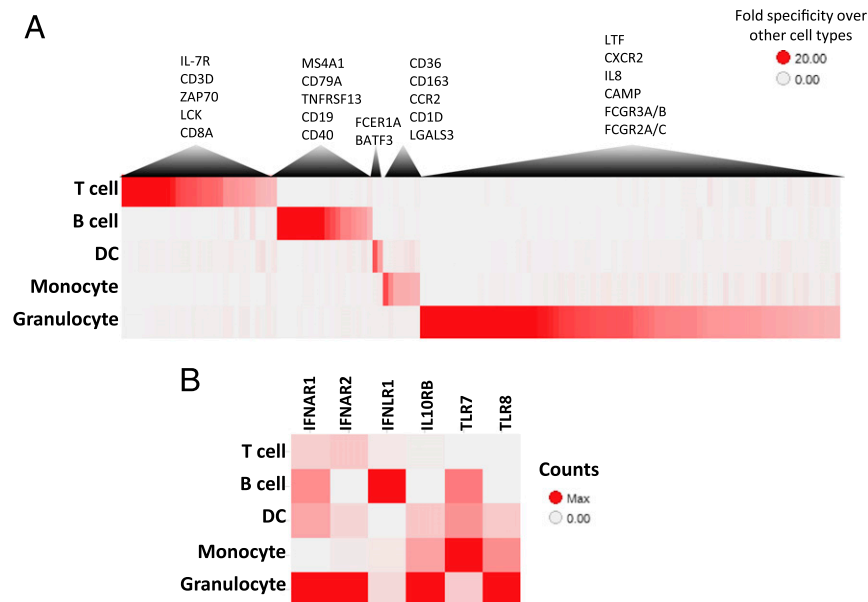


FIGURE 2. Gene expression analysis to characterize sorted cell types.

Cells were sorted from human blood by FACS into individual populations, and RNA was isolated and analyzed by NanoString using the Human Immunology Panel 2.0 (**A**). The fold change for each gene in each cell type versus the mean expression for all cell types was calculated, and the results are plotted as a heat map (**A**). Several genes found to be representative of each cell type are listed above the heat map. The expression of TLR7/8 and IFN pathway receptors in each cell type is shown (**B**) as a heat map with coloring based on the normalized counts for each gene in each cell type. The data are representative of two separate experiments testing two different donors.

SLE patient autoantibody and gene expression analysis. Blood samples were collected from healthy volunteers and SLE patients at the Mayo Clinic (Rochester, MN). Study participants had provided informed consent to participation in the study, and sampling was conducted using safe protocols approved by the Mayo Clinic IRB. Whole-blood samples were collected directly into PAXgene RNA Tubes and into sodium heparin tubes for isolation of plasma. RNA was extracted using the QIAGEN PAXgene RNA extraction kit and analyzed using NanoString and the TLR7/8 gene panel as described above. The log₂ fold change for the SLE patients compared with the healthy volunteer group was calculated. Plasma samples were profiled using a 128-Ag array (University of Texas Southwestern Microarray Core) to identify IgG autoantibody reactivities present in the patients and healthy controls as described elsewhere (<https://microarray.swmed.edu/products/product/autoantigen-microarray-super-panel-128-antigen-pan/>). The data from the array were analyzed as signal-to-noise ratios for each reactivity after normalization. To compare the levels of the different autoantibodies, z-scores were calculated for all the SLE patients relative to the mean of the healthy control group. A patient was considered positive for a reactivity if the z-score was >3. The gene expression data and the level of reactivity of each autoantibody for the SLE patients were then correlated by a Spearman correlation. The z-scores and log₂ fold changes were loaded into TIBCO Spotfire, and a Spearman correlation analysis was run, testing for correlation of all z-scores with all log₂ fold changes, and for each possible pairing, a rank R value and p value was determined.

RESULTS

Discriminating between direct TLR7/8 activation effects and secondary effects in whole blood

To investigate the different roles of TLR7 and TLR8 in immune responses and autoimmunity, we first characterized gene expression changes induced by their activation. Human whole blood was stimulated with the TLR7/8 dual agonist R848 for 1 or 24 h, and gene expression was measured using a custom 93-gene NanoString TLR7/8 panel containing a variety of genes expressed in immune cells representing different functions and pathways (Fig. 1). This same gene panel was also used in subsequent experiments. We selected genes for the panel known to be TLR7/8 regulated based on knowledge from prior experiments testing SLE patient samples, lupus mouse models driven by TLR7, and other in vitro studies of TLR7/8 activation. Blood was treated with R848 or rIFN- α in the presence or absence of the anti-IFNAR Ab anifrolumab, which blocks gene expression induced by type I IFNs. Anifrolumab was used as a tool to identify which genes are regulated secondarily by type I IFNs, such as IFN- α/β , as opposed to those regulated directly downstream of TLR7/8 activation. R848 treatment for 1 h resulted in gene expression changes that were not blocked by anifrolumab. In contrast, treatment with rIFN- α induced a separate set of genes, and their induction was completely suppressed by anifrolumab, indicating that these genes are very-sensitive IFN-response genes. At 24 h, the R848-induced genes included not only the genes induced at 1 h, but also canonical IFN-response genes such

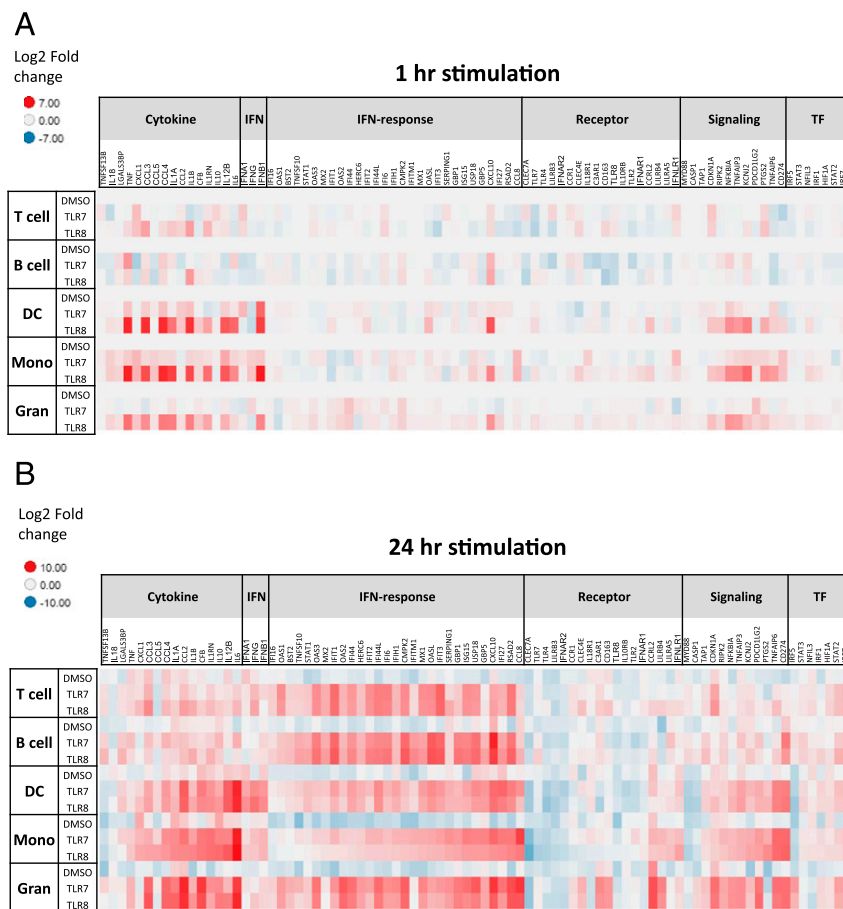


FIGURE 3. Treatment of whole blood with TLR-selective agonists and cell sorting identifies cell type-specific genes induced by TLR7/8 activation.

Human whole blood was treated with the TLR7-selective agonist CL-087 or the TLR8-selective agonist motolimod for 1 or 24 h, and then FACS was used to isolate individual cell types. RNA was purified from the sorted cells and analyzed for gene expression by NanoString. The heat maps show gene expression after 1 (A) or 24 h (B) of treatment, and coloring shows the log₂ fold change compared with the 1 h DMSO control for each cell type. The data presented are the average of two experiments using two separate donors. Gran, granulocyte; Mono, monocyte.

as *MX1* and *IFIT1*. The induction of the IFN-response genes by R848 was blocked by anifrolumab at 24 h, indicating that their induction was secondary to TLR7/8 activation and was instead due to the IFN- α/β induced at an earlier timepoint. Consistent with this model, *IFNB* was one of the genes highly induced by R848 at 1 h.

Characterization of the effects of TLR7 and TLR8 activation in specific cell types

The global effects of TLR7 and TLR8 activation in vivo are likely different because of their differential expression patterns and because they may have different downstream signaling pathway biases for IRFs and NF- κ B. To resolve how activation of these TLRs will ultimately result in different effects in vivo, we have taken a reductive approach and used TLR7- and TLR8-selective agonists and cell sorting to study gene expression changes after TLR activation in whole blood ex vivo.

Blood from healthy human volunteers was treated with the TLR7-specific agonist CL-087 (27) or the TLR8-specific agonist

motolimod (VTX-2337) (28), and then FACS sorted into five different populations of cells: granulocytes, T cells, B cells, monocytes, and DCs. The specificity of these ligands has been characterized previously, but we have provided data showing their selectivity for TLR7 or TLR8 using transfected HEK reporter cells (see Supplemental Fig. 2). The same experimental design was also used with R848 as a stimulus (data not shown). The DC population included both pDCs and mDCs as a first characterization of the DC family, and the subpopulations were later studied individually using intracellular cytokine analysis. Gene expression analysis of isolated RNA was performed using NanoString. First, the sorted cells were analyzed using the 594-gene Human Immunology Panel to characterize the sorted cell populations. This analysis identified genes that were selectively expressed in the various cell populations (Fig. 2A), and these genes consisted of many known lineage markers for the different cell types (e.g., *CD14* for monocytes, *CD3E* for T cells, etc.) and confirmed the robustness of the cell sorting. The expression of the relevant TLRs and IFNRs was also characterized using the

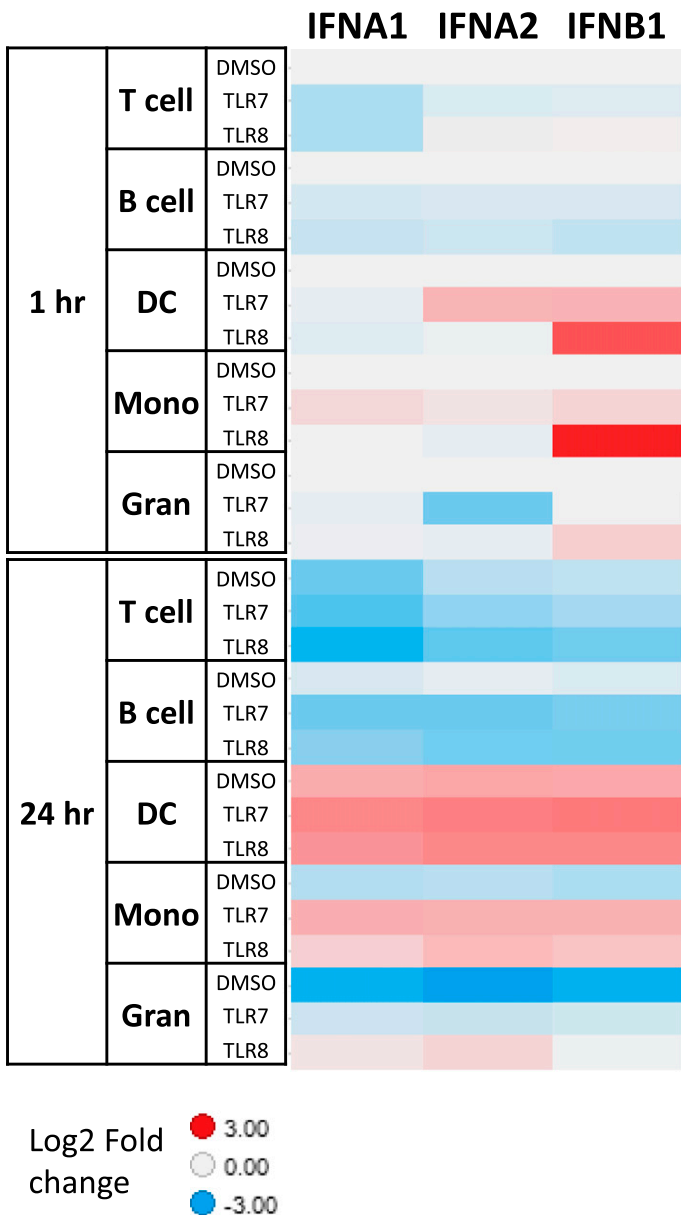


FIGURE 4. Analysis of IFN subtypes by quantitative PCR (QPCR).

To confirm the results of the NanoString analysis for IFN isoforms, the same RNA samples from Fig. 3 were analyzed by QPCR for expression of *IFNA1*, *IFNA2*, and *IFNB1*. *GAPDH* was measured as a housekeeping gene for normalization, and the fold change compared with the 1-h DMSO sample for each cell type was calculated. The data presented are the average of two experiments using two separate donors. Gran, granulocyte; Mono, monocyte.

TLR7/8 panel, and the results are presented (Fig. 2B). Our results indicate that the main cell types expressing TLR7 are B cells, DCs, and monocytes, whereas the cell types with the highest TLR8 expression are DCs, monocytes, and granulocytes.

Cells were also isolated from blood stimulated for 1 h (Fig. 3A) or 24 h (Fig. 3B) for gene expression analysis using the TLR7/8 NanoString panel (same gene panel used for Fig. 1), and the results

are presented as a heat map with the genes grouped by functional category. The functional categories for the genes were assigned by their accepted ontological classifications. After 1 h of stimulation, a subset of genes was induced in the DC, monocyte, and granulocyte populations, whereas few gene expression changes in T cells and B cells were found. The TLR8 agonist induced strong upregulation of multiple inflammatory cytokines such as *IL6*, *TNFA*, and *IL1B*. Several other signaling molecules are induced, such as *TNFAIP6*, *NFKBIA*, and *PTGS2*. Most of these gene changes are likely due to activation of the NF- κ B pathway, and indeed, some of them are well-recognized NF- κ B-regulated genes, such as *TNFA* and *IL6*. In contrast, the TLR7 agonist induced gene expression changes only in the DCs and monocytes and not in the granulocytes. TLR7 activation induced fewer genes and to a lesser extent than TLR8 activation. These patterns of TLR7 and TLR8 activation are consistent with the TLR expression patterns presented in Fig. 2B. At 1 h, *IFNB* expression is induced, but *IFNA* is not, nor is *IFNL*, which was below detectable levels and is not presented on the heat map. The expression of *IFNA1*, *IFNA2*, and *IFNB1* was confirmed by quantitative PCR (Fig. 4), as the NanoString counts were only slightly above the limit of detection.

Stimulation of blood for 24 h dramatically increased the number of genes induced in all cell types (Fig. 3B). Also, at 24 h gene induction is now noted in T and B cells. Gene induction in these two cell types is most dramatic for the IFN-response genes, suggesting that these cells are being activated secondarily by the IFN produced earlier by other cell types. The DCs, monocytes, and granulocytes also showed induction of the IFN-response genes at 24 h, indicating that they were also responding to IFN produced earlier. These three cell types also showed an induction of a greater number of cytokines, chemokines, and other receptors and signaling molecules at 24 h compared with at 1 h. To illustrate how these cell types were responding to TLR7 versus TLR8 activation, plots were constructed comparing the results for each TLR after 1 h of stimulation (Fig. 5). The results show that TLR8 activation more strongly induced NF- κ B-regulated genes such as *IL1B* and *IL6* in all these cell types, with TLR7 being responsible primarily only for *IFNA* and *IFNB* production in DCs. Overall, these results indicate specific direct effects of TLR activation in some cell types, whereas secondary effects are seen at later timepoints in a broad range of cells.

Intracellular staining to characterize single-cell cytokine expression after TLR7/8 activation

To study TLR activation in subsets of the monocyte and DC populations on a single-cell level, flow cytometry analysis of intracellular cytokine expression was used. Human PBMCs were stimulated with the TLR7 and TLR8 agonists, and after 4 h, the cells were analyzed for intracellular TNF- α , IL-6, IL-1 β , and IFN- α expression (Figs. 6A, 6B, 7). Consistent with the gene expression results, T and B cells did not significantly respond to treatment with either TLR agonist. Only pDCs were found to be producers of IFN- α , and only a subset of that cell type was producing the cytokine. Furthermore, the cytokine was only produced in response to TLR7 stimulation. pDCs also produced TNF- α and

IL-1 β after TLR7 activation, but did not produce IL-6. Monocytes were highly stimulated by TLR8 activation and produced all the cytokines measured except for IFN- α , and nearly the entire monocyte population responded homogeneously. Interestingly, monocytes responded to TLR7 activation by producing large amounts of IL-1 β , but not the other cytokines. mDCs showed a response pattern similar to the monocytes but with a smaller fraction of the population producing cytokines. Results were compared for four donors, and the response was found to be very consistent from donor to donor (Fig. 6B). Intracellular cytokine production after 24 h of stimulation was also measured, and the results were similar to those for 4 h (Supplemental Figs. 3, 4), with the exceptions that IL-6 levels are higher and IFN- α production has decreased in pDCs.

To assess on a single-cell level if the response differed from cell to cell within each population, a viSNE analysis was performed on the 4 h data for the myeloid cell populations. The myeloid cell types were subdivided for this analysis by including the markers CD1c and CD16 to further fractionate the mDCs and monocytes, respectively. A diagram defining the major cell populations and the expression of the subsetting markers is shown (Fig. 7). For the pDCs, it was found that the portion of the population producing IFN- α was also the subset producing TNF- α , whereas the remainder of the cells were unresponsive. Similarly, the mDCs producing IL-1 β were the same cells producing TNF- α and IL-6. The mDCs activated by TLR7 to produce IL-1 β were the same cells activated by TLR8 to produce that cytokine. However, the same cells activated by TLR8 were not activated by TLR7 to produce the other cytokines. The CD1c-positive and -negative mDC fractions behaved similarly, and that marker did not seem to mark populations responding differently. The CD14⁺ monocyte population almost uniformly was activated by TLR8 to produce IL-1 β and IL-6, but there were cell-to-cell differences in the expression levels of TNF- α . Interestingly, the same monocytes producing higher levels of TNF- α in response to TLR8 activation were also the few monocytes that responded to TLR7 stimulation. The intracellular cytokine analysis was also performed after overnight stimulation, and the results are shown in Supplemental Figs. 3, 4. With overnight stimulation, there were similar results but a more uniform expression of cytokines within each cell type.

Overall, the intracellular cytokine staining results indicate that there are not only differences in the responses of the different myeloid populations, but also between cells within each population, as some cells are highly responsive, whereas others are unresponsive. The differences could be due to different levels of expression of the TLRs between cells or that some cells are refractory for other reasons.

Regulation of TLR7/8-induced genes in lupus mice

TLR7 and TLR8 have been implicated to play a role in autoimmune disease, and in lupus in particular. To better understand the expression of TLR7-regulated genes in lupus, we have used the pristane mouse lupus model, which is known to be driven by TLR7 and IFN (17, 18). Furthermore, a strong IFN gene signature is present in these mice similar to the IFN gene signature found in

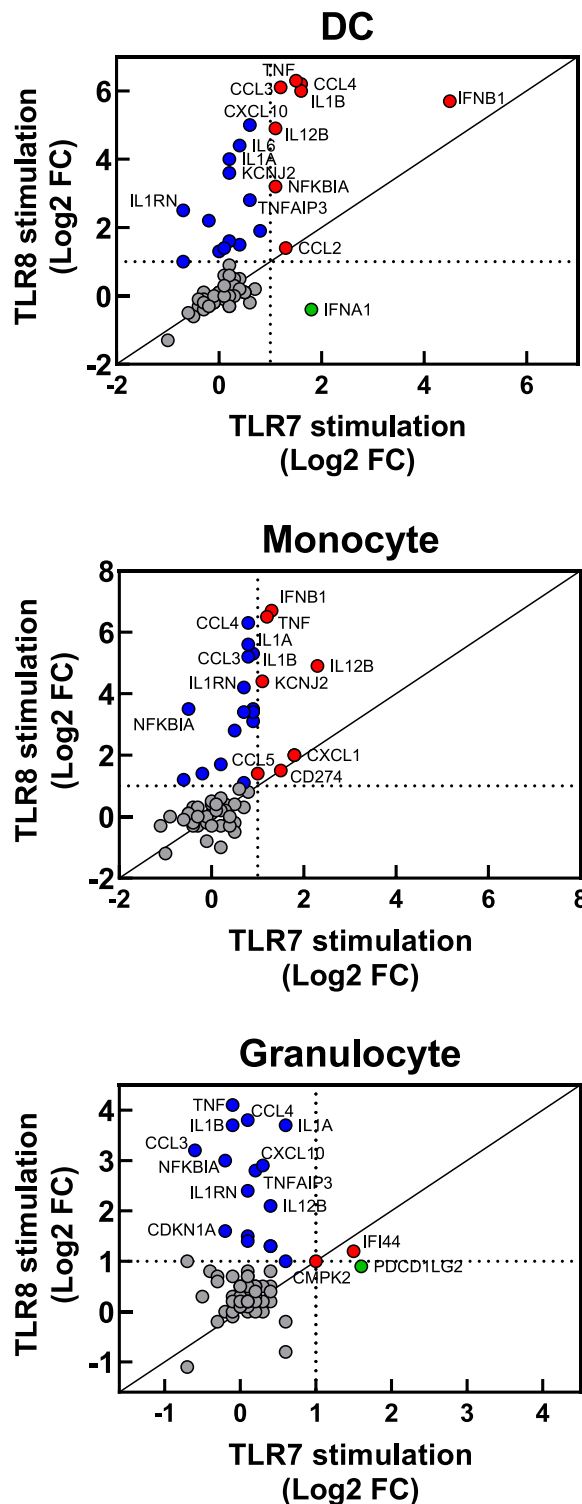


FIGURE 5. Correlation of TLR7 and TLR8 stimulation effects on gene expression in myeloid cells.

The log₂ fold change induced by TLR7 or TLR8 activation is plotted for the three most highly activated cell types from Fig. 3. Genes induced specifically by TLR7 are colored green, by TLR8 specifically are colored blue, and by both TLRs are colored red. The data presented are the average of two experiments using two separate donors.

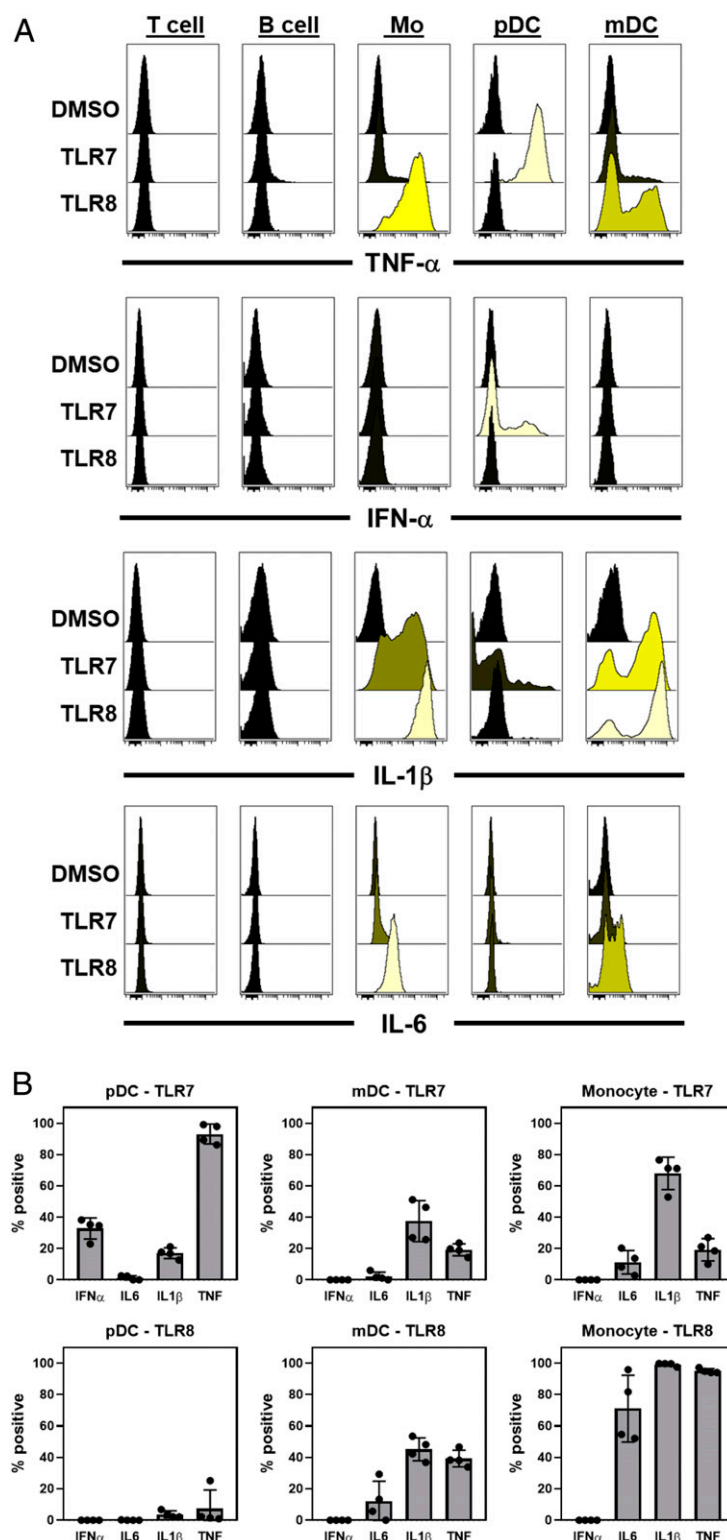


FIGURE 6. Flow cytometry analysis of intracellular cytokine expression after TLR7 and TLR8 activation.

Human PBMCs were treated with brefeldin A and then stimulated with the TLR7-selective agonist CL-087 or the TLR8-selective agonist motolimod for 4 h and then analyzed for intracellular cytokine expression by flow cytometry. The expression of cytokines in different cell populations is shown as histograms (A) for one representative donor for five different cell populations. The percentage of each cell population staining positive for each cytokine was determined, and the results for four donors from two separate experiments are presented (B). Each individual donor is represented by a dot, and the group means and SD are represented as bars. Mo, monocyte.

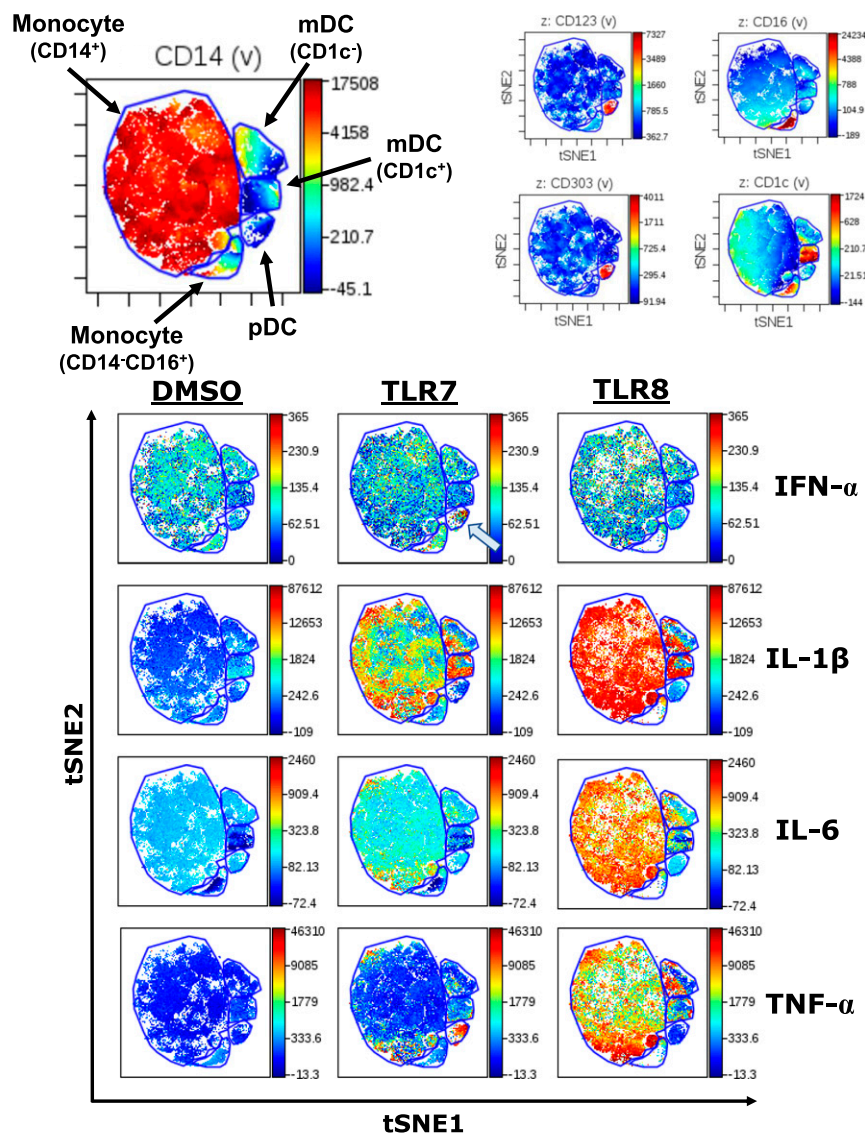


FIGURE 7. viSNE analysis of flow cytometry intracellular cytokine staining.

To study the expression of cytokines in individual cells in different populations, viSNE analysis was performed on the data from Fig. 6, and representative results from one of the four donors tested are presented. The different cell populations are gated and labeled, and the intensity of staining for each subset identifying marker and cytokine is shown by coloring.

many lupus patients (29). The role of TLR8 in driving disease in these mice has not been defined, and its study in mice is complicated, as the presence of active TLR8 in mice is controversial. To identify genes associated with disease, the expression of which might be driven by TLR7 directly or IFN secondarily in these mice, we used the Ab MARI-5A3 (30) that blocks the IFNAR similar to anifrolumab, which has been investigated in SLE clinical trials (31). Disease was induced in DBA/1 mice by injection with pristane and at 2 mo after injection treatment was initiated with the anti-IFNAR Ab. At 1, 4, or 8 wk of treatment, blood samples were collected, and a mouse surrogate of the TLR7/8 NanoString panel was used for gene expression analysis. It was found that a large number of the genes induced by TLR7/8 activation in humans were increased with disease in

the mice (Fig. 8A). The anti-IFNAR Ab completely reversed the induction of IFN-regulated genes, such as *OAS1* and *IFI44*, within 1 wk of treatment. However, the expression of primarily NF- κ B-regulated genes, such as *IL1B* and *IL6*, was virtually unchanged with treatment. Of the genes increased >1 log₂ fold change with disease (Fig. 8B), there was a group that was strongly affected by anti-IFNAR and a group not impacted by treatment. To provide a quantitative illustration of this differential effect, an IFN gene signature score was calculated for each mouse using 22 canonical IFN-regulated genes, and an NF- κ B gene signature score was also calculated using five canonical NF- κ B genes (*CSF1*, *IL1B*, *IL6*, *NFKB1A*, and *TNF*). The IFN scores were strongly reduced by anti-IFNAR, but the NF- κ B scores were unaffected (Fig. 8C). These results suggest that inhibiting IFN activity alone

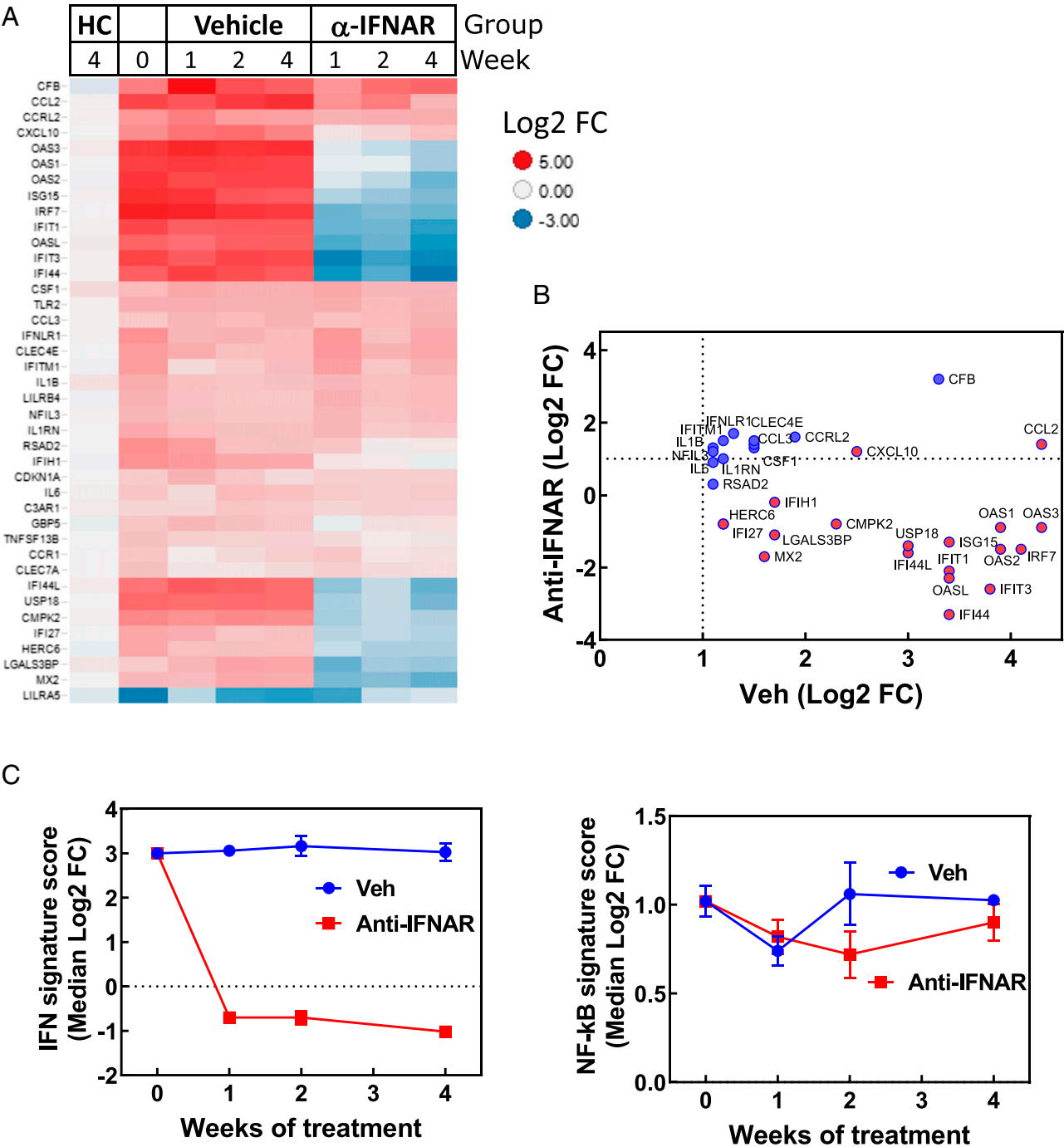


FIGURE 8. Anti-IFNAR treatment reduces a subset of disease genes in lupus mice and defines specifically IFN-regulated genes. Lupus-like disease was induced in DBA/1 mice by injection of pristane i.p. At 8 wk after pristane injection, mice were treated with an anti-IFNAR-1 Ab (α -IFNAR) or PBS (Vehicle) three times per week. After 1, 2, or 4 wk of treatment, blood samples were collected, and RNA was isolated for gene expression analysis by NanoString. The log₂ fold change in expression was calculated for each mouse compared with a nonpristane-injected healthy control (HC) group. The heat map (A) shows the log₂ fold change for the group mean of each condition ($n = 4$). The genes increased >1 log₂ fold change by disease at week 4 are shown in a plot for the mean of the vehicle group versus the mean of the α -IFNAR group (B). Genes significantly affected by α -IFNAR treatment are shown in purple. An IFN score and an NF- κ B score were calculated for each mouse using canonical genes for each pathway, and the group means are plotted (C). The data shown are from one experiment with four mice per group and are representative of two separate experiments.

TABLE I. Association of autoantibody reactivity with IFN status

Autoantibody	IFN Hi Autoantibody+ (n)	IFN Lo Autoantibody+ (n)	IFN Hi Autoantibody+ (%)	IFN Lo Autoantibody+ (%)	p Value
Nucleosome Ag	24	4	85.7	36.4	1.41×10^{-3}
ssRNA	19	2	67.9	18.2	4.20×10^{-3}
Chromatin	21	3	75.0	27.3	4.90×10^{-3}
Collagen VI	17	2	60.7	18.2	1.61×10^{-2}
Heparin	16	2	57.1	18.2	2.81×10^{-2}
Genomic DNA	17	3	60.7	27.3	6.25×10^{-2}
U1-snRNP-C	13	2	46.4	18.2	1.08×10^{-1}
Ro-52/SSA	10	7	35.7	63.6	1.20×10^{-1}
dsDNA	24	7	85.7	63.6	1.31×10^{-1}
Ribo phosphoprotein P0	12	2	42.9	18.2	1.56×10^{-1}
ssDNA	13	4	46.4	36.4	5.80×10^{-1}
PM/Scl-100	14	5	50.0	45.5	8.05×10^{-1}

Autoantibody+ defined as z-score >3.

may not be sufficient to completely suppress the consequences of TLR7 activation. However, it has been found that IFNAR KO in this model does have a dramatic effect (17, 18, 32), and this is consistent with the postulated strong role for IFN in driving human SLE.

Correlation of TLR7/8-regulated gene expression and autoantibodies in SLE patients

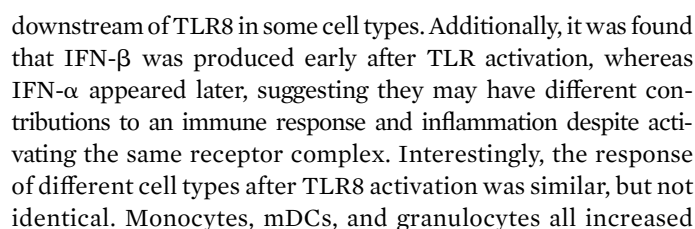
Part of the supporting evidence for the involvement of TLR7 and TLR8 in driving SLE pathogenesis is the presence of RNA and RNA-binding protein-specific autoantibodies in the circulation of lupus patients, which may serve as activators of TLR7/8 when they are taken up into cells as immune complexes (33). Thus, we tried to correlate the expression of TLR7/8-regulated genes with the presence of different autoantibodies in the blood of lupus patients. Samples were collected from 39 SLE patients and 11 healthy controls and tested for autoantibody reactivity using an array containing 128 Ags and for gene expression using the same TLR7/8 panel used in our prior experiments. Data for expression of 22 IFN-regulated genes were used to calculate an IFN score for each patient, and a cutoff was set for defining the SLE patients as IFN high (IFN Hi) or IFN low (IFN Lo) (data not shown). The autoantibody array analysis identified 12 reactivities that were more prevalent in the SLE patients compared with the healthy control group. The percentage of the IFN Hi and IFN Lo patients that were positive for each of these reactivities was then calculated (Table I), and five of these reactivities were found to be statistically different ($p < 0.05$) in the percentage positive for the IFN Hi and IFN Lo populations (nucleosome Ag, ssRNA, chromatin, collagen VI, and heparin). These results suggest that RNA-binding reactivity may be driving some of the IFN activity found in lupus patients but that other nucleic acid receptors may play a role as well.

We next drilled down to a single gene level to try and correlate expression of each gene with autoantibody reactivity. The genes that were found to be significantly different between the SLE and healthy control groups were identified and are shown in a heat map (Fig. 9A). The expression of each of these genes and the level of reactivity of each autoantibody was then correlated by a Spearman correlation, and the R values and p values are shown

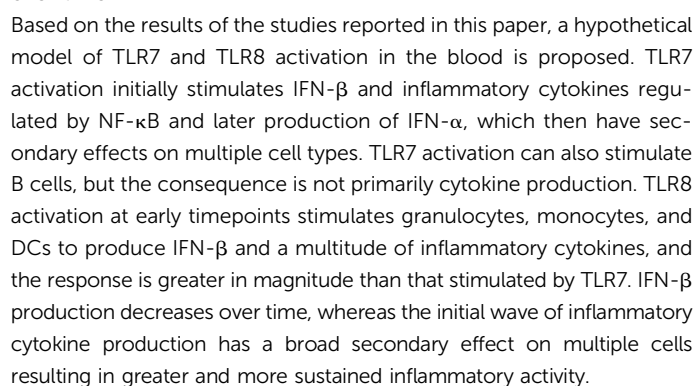
for each gene (Fig. 9B). On a gene level, there was a poor correlation of autoantibody reactivity with gene expression. There were a few genes that showed significant correlations ($p < 0.01$) with autoantibodies, such as *BST2* with nucleosomes; *C3AR1* with U1-snRNP-C; *GBP1*, *SERPING1*, and *STAT1* with ssRNA; and *STAT2* with ribosomal phosphoprotein P0. However, in all of these cases, the R value was below 0.4, so the correlation was not very strong. Although on a global level there does seem to be a correlation between nucleic acid binding reactivities and IFN-regulated gene expression, consistent with some prior published results (23), on a single gene level the correlation is not very strong. The association is more robust when using a binary classification (positive or negative) for the autoantibody reactivity (data not shown). Interestingly, the best correlation for almost every gene was either RNA/DNA or some nucleic acid binding protein, so it seems entirely possible that nucleic acid sensor activation plays a role in driving IFN production in these patients. Taken together, our results suggest a model by which TLR7 and TLR8 activation in different cell types may contribute to the evolution of an immune response or autoimmunity in the case of SLE (Fig. 10).

DISCUSSION

To date, much more is known about TLR7 biology in comparison with TLR8 biology. This may be due, in part, to the challenges associated with studying TLR8 in mice. Thus, it is possible the role of TLR8 as a contributor to immunity and autoimmune diseases has been underappreciated. Our results suggest that activation of TLR8 may drive strong production of NF- κ B-dependent inflammatory cytokines, particularly from granulocytes, mDCs, and monocytes. Given the higher numbers of granulocytes compared with other cell populations in the circulation, TLR8 may have a large inflammatory effect by stimulating granulocyte activation. Although TLR7 and type I IFN are considered key drivers of lupus pathogenesis, TLR8 activation may play a role in end organ disease because of the strong production of cytokines, such as TNF- α and IL-1 β . Although we did not find that TLR8 activation induces IFN- α production, it was noted that IFN- β was produced



Cell type-specific responses to TLR7 activation were also noted, particularly between B cells and pDCs. pDCs responded to TLR7 activation with a marked increase in cytokine production and IFN- α . It has been previously reported that B cells respond to TLR7 stimulation with proliferation, increased IgG production, and class switching (34). However, our experiments were not designed to measure those readouts, and thus, very little B cell response to TLR7 activation was captured by the gene expression panel used in our studies. Overall, our results suggest real differences in the signaling downstream of the TLRs, with TLR7



showing a dual IRF/NF- κ B activation and TLR8 showing a bias toward NF- κ B activation and stronger activation of this pathway relative to TLR7. These findings are consistent with data from earlier studies, suggesting a similar differentiation between TLR7- and TLR8-stimulated cytokine production from different cell types (11).

In exploring how TLR7 and TLR8 may contribute to immunity and autoimmunity, the timing of downstream responses to activation of the TLRs and the long-term consequence of their activation *in vivo* are important to consider (see Fig. 10). In the studies presented in this paper, the immediate direct effects of TLR7/8 activation (1 h) were limited but were expanded and amplified with time (24 h). Additionally, differences in some of the cytokine profiles changed with time, particularly for the IFNs with IFN- β expressed immediately whereas IFN- α production was delayed. How TLR7- and TLR8-stimulated responses *in vivo* differ at timepoints beyond 24 h is difficult to predict. At 24 h in sorted cells, we found an amplified inflammatory response and robust IFN gene signature, but in many cell types, expression of TLR7, TLR8, IFNAR1, and IFNAR2 are decreased indicating a feedback loop may be downregulating the response. The outcomes of constant TLR7/8 activation in a disease setting such as lupus with more sustained TLR activation may be different from in a cell culture situation with isolated cells from healthy donors. In the pristane lupus mouse model, we found highly elevated expression of IFN-response genes and increased expression of numerous other NF- κ B-driven inflammatory genes. Thus, it seems likely that feedback mechanisms that might normally self-limit a normal immune response to TLR activation cannot control inflammation in an autoimmunity setting. Treatment of the mice with the anti-IFNAR Ab rapidly and effectively reduced the expression of IFN-response genes, suggesting that there is constant and ongoing TLR activation and IFN production in lupus. Reports suggest epigenetics may play a role in promoting and perpetuating the expression of IFN-response genes, even in the absence of high IFN levels (35, 36). Conversely, the data reported in this study suggest that sustained elevated IFN-response gene expression is more dependent on constant levels of elevated IFN. The results of this pristane lupus mouse experiment can likely be translated to humans as evidence that IFN activation is an ongoing process subsequent to TLR activation. However, extrapolation of our mouse results to human is limited in that the relative contributions of either TLR7 or TLR8 to the development of lupus cannot be determined because of the lack of TLR8 activity in mice and the situation may be different from that in humans.

The rate of onset and efficacy by which the anti-IFNAR Ab reduced the IFN gene signature in pristane mice was impressive and supports the continued evaluation of mechanisms for IFN blockade to reduce SLE disease in humans. However, the anti-IFNAR Ab did not affect the expression of NF- κ B-dependent inflammatory genes in our mice. If the NF- κ B inflammatory genes are key contributors to disease pathogenesis, then IFN blockade may be insufficient as monotherapy. However, the mouse anti-IFNAR Ab has shown efficacy in BXS β -Yaa mice that have TLR7-driven disease (30), and IFNAR KO does reduce disease in the

pristane lupus model (32). Two anti-IFN- α Abs (sifalimumab and rontalizumab) and an anti-IFNAR Ab (anifrolumab) have been tested in clinical trials for SLE as well as other diseases. Sifalimumab showed neutralization of the IFN gene signature, as did rontalizumab, and sifalimumab showed impressive efficacy (37–41). Anifrolumab showed efficacy in a phase 2 SLE trial (31) but failed to meet its primary end point (SRI-4) in one phase 3 trial (TULIP 1), but in a separate phase 3 trial, a different primary end point (BILAG-Based Composite Lupus Assessment [BICLA]) was met. SLE is a notoriously challenging disease to treat, and the reasons for the mixed results in these trials may be numerous, including the fact that it appeared that IFN neutralization was incomplete, variable, or more than IFN was driving disease. SLE is a highly complex and heterogeneous disease, and it is likely that targeting more than one pathway is required to achieve high efficacy.

There is also evidence suggesting that TLR7 and TLR8 may be differential drivers of SLE and thus attractive drug targets. However, development of TLR7- and TLR8-specific inhibitors has produced few candidate drugs. Hydroxychloroquine is an antimalarial drug frequently used to treat SLE with modest efficacy, and part of its mechanism of action may be inhibition of endosomal TLR signaling (14). A TLR7/8/9 inhibitor oligonucleotide (IMO-8400) was tested in a phase 2 psoriasis clinical trial in which it showed some efficacy (42), but it failed to meet the primary end point in a phase 2 dermatomyositis trial. IMO-8400 has not been tested in lupus patients yet. The fact that oligos were employed to block these receptors underscores the challenges of developing small molecules or Abs targeting them. New small molecule TLR7/8 inhibitors reported in the patent literature may provide greater activity. Because lupus is a heterogeneous disease, it is likely that not all patients have TLR7 or TLR8 driving their disease, and patient stratification to select patients for TLR7/8-inhibitor treatment may be necessary if not all patients respond to inhibitor treatment. We demonstrated that a number of genes known to be downstream of TLR7/8 activation had increased expression in SLE patients compared with healthy individuals and were also increased in lupus mice; we hypothesized that these same SLE patients might also have higher titers of autoantibodies likely to activate TLR7/8. However, the correlation of higher expression of the genes and autoantibodies was weak. Our results do not indicate that RNA-binding autoantibodies are an obvious stratification marker for selecting patients with potential TLR7/8-driven disease, and it could be the case that TLR7/8 activation occurs in SLE patients by microRNAs or other RNA molecules delivered via exosomes (7, 8). Nevertheless, TLR7/8 inhibition could be a promising therapeutic strategy for interrupting overactive IFN and NF- κ B signaling in some autoimmune disease patients in whom these pathways are dysregulated. Given that TLR7 and TLR8 have different cell type expression patterns and different activation outcomes, a dual TLR7/8 inhibitor may provide greater efficacy by reducing both type I IFN- and NF- κ B-driven inflammation. In summary, the results reported in this study provide new insight into TLR7/8 biology and a rationale for studying a new therapeutic approach that could potentially benefit patients with autoimmune disease.

DISCLOSURES

The following authors were all employed at EMD Serono Research and Development Institute during the time this manuscript was prepared, and some of these authors own stock in the company: A.T.B., E.T., A.P., S.K., M.M.P., J.V., Y.W., and S.L.O. The following authors have served as consultants to EMD Serono Research and Development Institute and received more than \$5,000 in research support in the past year: T.B.N. and M.A.J.

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