Impact of the Type I Interferon Receptor on the Global Gene Expression Program During the Course of Dendritic Cell Maturation Induced by Polyinosinic Polycytidylic Acid

Amy L. Olex,^{1,*} William H. Turkett,¹ Kristina L. Brzoza-Lewis,^{2,†}
Jacquelyn S. Fetrow,^{1,3,‡} and Elizabeth M. Hiltbold⁴

Dendritic cell (DC) maturation involves widespread changes in cellular function and gene expression. The regulatory role of IFNAR in the program of DC maturation remains incompletely defined. Thus, the time evolution impact of IFNAR on this process was evaluated. Changes in DC phenotype, function, and gene expression induced by poly I:C were measured in wild-type and IFNAR^{-/-} DC at 9 time points over 24 h. Temporal gene expression profiles were filtered on consistency and response magnitude across replicates. The number of genes whose expression was altered by poly I:C treatment was greatly reduced in IFNAR^{-/-} DC, including the majority of the downregulated gene expression program previously observed in wild-type (WT) DC. Furthermore, the number of genes upregulated was almost equal between WT and IFNAR^{-/-} DC, yet the identities of those genes were distinct. Integrating these data with protein–protein interaction data revealed several novel subnetworks active during maturation, including nucleotide synthesis, metabolism, and repair. A subnetwork associated with redox activity was uniquely identified in IFNAR^{-/-} DC. Overall, temporal gene expression and network analyses identified many genes regulated by the type I interferon response and revealed previously unidentified aspects of the DC maturation process.

Introduction

DENDRITIC CELLS (DCs) are central mediators of immunity and hold significant promise as vaccines or therapies for cancer and a variety of diseases (Steinman 2008; Trumpfheller and others 2012; Matos and others 2013). DC maturation is a pivotal event in the development of adaptive immune responses. During this transformational process, DCs respond to pathogen or inflammatory insult by up- and/or downregulating the expression of a wide range of proteins and genes. Some of the most well-documented features of DC maturation include decreased endocytosis and membrane turnover, enhanced antigen degradation, increased cell surface expression of MHC-peptide complexes and costimulatory molecules, secretion of proinflammatory cytokines, and upregulation of chemokine receptors, adhe-

sion molecules, and cytoskeletal molecules required for cellular migration to lymphoid organs (Steinman and others 1997). Ultimately, these changes in gene expression and intracellular transport greatly enhance the capacity of DC to activate naive T cells.

The type I interferon response has long been known to be a key factor in the innate immune response to viruses (and likewise in the response to polyinosinic acid–polycytidylic acid, poly I:C) (Baron and others 1969; De Clercq and others 1970; Hilleman 1970; Schafer and Lockart 1970). Yet, little is known about the downstream effects of this response on the global gene expression program of DC maturation induced by viruses. While genes that are upregulated during DC maturation—such as costimulatory molecules and cytokines—are known hallmarks of the maturation process (Steinman and others 1997), our previous studies have shown that the overall

¹Department of Computer Science, Wake Forest University, Winston-Salem, North Carolina.

²Department of Microbiology and Immunology, Wake Forest School of Medicine, Winston-Salem, North Carolina.

³Department of Physics, Wake Forest University, Winston-Salem, North Carolina.

⁴Department of Biological Sciences, Auburn University, Auburn, Alabama.

*Current affiliation: Center for Clinical and Translational Research, Virginia Commonwealth University, Richmond, Virginia.

†Current affiliation: Department of Biology and Geosciences, Clarion University, Clarion, Pennsylvania.

‡Current affiliation: Department of Chemistry, University of Richmond, Richmond, Virginia.

pattern of changes in gene expression induced by the viral RNA mimetic, poly I:C, consists predominantly of down-regulation (Olex and others 2010). However, the underlying control mechanisms of this response are still unknown; thus, we set out to determine how the gene expression dynamics we had previously observed in wild-type (WT) DC would be altered in the absence of the type I interferon response to better understand how it impacts the cellular function of DC in T-cell activation.

Poly I:C is an attractive candidate as an antiviral/immunomodulatory therapeutic due to its potent activity in stimulating a strong type I interferon response. It is known to activate this response through TLR3/TRIF and/or cytosolic receptors such as RIG-I and MDA-5 (Jiang and others 2003; Oshiumi and others 2003; Wietek and others 2003; Gitlin and others 2006; Hausmann and others 2008). The first phase of the response is known as induction and results in the expression of interferon beta, among other genes (Matsumoto and Seya 2008). The second phase of the type I interferon response, in which nascent interferon beta binds to the type I interferon receptor (IFNAR) and initiates an additional wave of gene expression, is known as signaling (Bonjardim and others 2009). During the signaling phase, expression of many genes is induced, including genes distinguished by a common motif present in their promoters, an Interferon Signaling Responsive Element (ISRE) (Kerr and Stark 1991). Many of these genes are known to play a role in antiviral immune responses and viral replication inhibition (Matsumoto and others 2004). In addition, the type I interferon response is key to regulation of many MHC class I genes (Pascucci and others 1988), where antigen presentation by these molecules is one of the crucial functions of DC, leading to cytolytic T-cell stimulation; however, the roles of many interferon-regulated genes in the DC maturation response remain undefined.

In this study, we aimed to determine which aspects of the DC maturation response stimulated by poly I:C were regulated by type I interferon signaling. We chose to use murine bone marrow-derived GM-CSF-driven inflammatory DC (GMDC) for this study as these cells serve as a well-characterized experimental model system. GMDCs are also in development as vaccines for a variety of diseases, most notably cancer (Pyzer and others 2014). We have previously shown that GMDC responds to Poly I:C stimulation with both a strong type I interferon response as well as a strong maturation response, rendering these cells potent inducers of T-cell activation (Ahmed and others 2006, 2009).

First, we performed a targeted analysis of DC maturation and T-cell priming function to determine the extent to which the type I interferon response impacted basic DC functions. Our next objective was to obtain a comprehensive view of gene expression dynamics during DC maturation in the presence and absence of IFNAR using DC generated from mice lacking IFNAR (IFNAR^{-/-}). Our approach was to use microarrays to measure gene expression changes at 9 time points over 24 h following stimulation. We observed that the number of genes with altered gene expression by poly I:C treatment in wild type was greatly reduced in cells lacking IFNAR. Surprisingly, the greatest change in the overall gene expression program in IFNAR^{-/-} DC was the decrease in the number of genes whose expression was downregulated. Another major difference was that while a similar number of genes were upregulated in both WT and IFNAR^{-/-} DC, the

gene identities and temporal profiles were different. Thus, while the majority of the gene expression response to poly I:C could be considered IFNAR dependent, a distinct gene expression program was induced in cells lacking this receptor. IFNAR appears to be responsible for the signaling that results in the expression downregulation of the cohort of genes induced by poly I:C during DC maturation. Finally, we integrated our temporal gene expression data with protein-protein interaction data and performed a network analysis of both WT and IFNAR^{-/-} results. Active subnetworks of genes that are up- or downregulated were identified, opening potential avenues for future research.

Materials and Methods

Mice

Female C57BL/6 mice (8–16 weeks old) purchased from Taconic (Taconic Farms, Inc.) were used as a source of wild-type (WT) bone marrow. Age-matched mice lacking the type I interferon receptor (IFNAR^{-/-}) were acquired from Dr. Christian Schindler and backcrossed onto the C57BL/6 background for a total of 8 generations.

DC isolation

DCs were generated by culture of bone marrow with GM-CSF over a 6–7-day period as described previously (Brzoza and others 2004). These DCs are representative of immature inflammatory DC *in vivo* and express CD11c, CD11b, and low levels of costimulatory molecules at the time of treatment. To exclude any contaminating monocytes or progenitor cell types, CD11c-positive selection was performed upon harvested cells before Poly I:C treatment. Three biological replicate experiments were performed for each time course. For each biological replicate, RNA from DCs of 3 mice was combined. Collectively, 9 sets of DCs were grown, treated, and subjected to gene expression analysis from both WT and IFNAR^{-/-} mice.

Measurement of cytokines and costimulatory molecules

DCs were plated in 48-well plates at a density of 5×10^5 DC/mL. The cells were then treated with Poly I:C (20 µg/mL) and incubated for 24 h. Culture supernatants were then removed, and the production of cytokines (IL-6, IL-12, TNF-α, or IFN-β) was measured by ELISA (OptEIA kits, BD Pharmingen, IFN-β ELISA kit was purchased from Life Technologies). The cells were then harvested and stained with fluorescently conjugated antibodies to murine CD40, CD80, CD86, and H-2K^b. Nonspecific background staining was assessed using isotype control antibodies (data not shown). DCs were costained with antibodies to CD11c to enable analysis of costimulatory molecules expressed specifically by the DC population. Statistical significance was determined using a 2-tailed, paired Student's *t*-test. *P* values of less than 0.05 were considered significant differences.

Measurement of antigen processing, presentation, and T-cell activation

DCs (2×10^4 /mL, 100 µL/well) were plated in 96-well plates and left untreated, treated with Poly I:C (20 µg/mL),

or infected with a recombinant strain of *Listeria* that expresses the model antigen ovalbumin *LmOVA* at the indicated multiplicity of infection (MOI). *LmOVA* was the gift of Dr. Hao Shen (Pope and others 2001). For cells treated with Poly I:C, exogenous OVA peptide (SIINFEKL) was added to the culture before T-cell addition at 1 ng/mL. The cells were cultured for 24 total hours before the addition of T cells. Naive, OVA-specific CD8⁺ T cells were isolated from the spleens of OT-1 TCR transgenic mice (Jackson Laboratory). T cells were stained with CFSE according to the manufacturer's instructions and were added to the DC at a ratio of 10 T cells per DC. T cells were harvested 72 h later, and the production of IFN- γ was then measured by incubation with OVA (257–264) peptide (1 μ g/mL) in the presence of GolgiPlug reagent for 5 h. The cells were then stained, fixed, and permeabilized for intracellular cytokine staining using the Cytotfix/Cytoperm kit for from BD Biosciences according to the manufacturer's instructions. Flow cytometric data were acquired using a BD FACSCalibur (BD Biosciences). CFSE proliferation data were quantitated using the FLOWJO program for flow cytometric data analysis (Tree Star, Inc.). The Division Index represents the mean number of divisions that a cell present in the starting population undergoes. Statistical significance was determined using a 2-tailed, paired Student's *t*-test. *P* values of less than 0.05 were considered significant differences.

RNA isolation/purification

Immature DCs (10⁷/well in 5 mL) were cultured on 100-mm dishes and treated with 20 μ g/mL poly I:C (InvivoGen) for 0 (control), 1, 2, 3, 6, 9, 12, 18, or 24 h. For each sample replicate, the purified RNA from 3 mice was combined just before hybridization on microarray chips. Culture media were then gently removed, and RNeasy lysis reagent (Qiagen) was added to preserve RNA. Any nonadherent DCs that were harvested with supernatants were spun down, resuspended in RNA later, and recombined with adherent cells from the same sample. Cells were stored until completion of experiment, and RNA was purified from all samples in each replicate simultaneously. RNA purification was carried out using RNeasy spin columns (Qiagen) according to the manufacturer's instructions.

RNA hybridization and data preprocessing

Microarray processing and data normalization were completed by the microarray core laboratory in the Comprehensive Cancer Center at Wake Forest Health Sciences. Three biological replicates of each time course were performed for WT and IFNAR^{-/-} DC with equal amounts of RNA from 3 individual mice pooled per time point. Wild-type and IFNAR^{-/-} replicates were not pair matched. Purified RNA was hybridized to Affymetrix mouse 430 2.0 nucleotide chips at the previously indicated time points. Array images were processed using the Affymetrix AGCC software, and raw data were normalized across all replicates and time points using systematic variation normalization (Chou and others 2005). Reported data included the log₂ of the signal intensity and the detection *P* value, which was calculated by Affymetrix AGCC software.

Time course relative gene expression changes were calculated for WT and IFNAR^{-/-} data sets independently. The

average signal intensity of the 3 replicates from time zero for each cell type (WT or IFNAR^{-/-}) was used as control for the respective time course. Relative expression for each cell type was calculated as the signal log ratio (SLR) of the average control intensity to the intensity of each replicate at each time point for the respective cell type (ie, WT time zero was used as the control for WT data and IFNAR^{-/-} time zero was used as control for IFNAR^{-/-} data).

Differential expression filtering of microarray data

The WT and IFNAR^{-/-} time course data sets were each filtered to identify significantly and consistently regulated genes during DC maturation. Filtering was performed as previously described (Olex and others 2010) with a few alterations to accommodate the 3 biological replicates (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/jir). Briefly, each replicate time course was first filtered independently on the Affymetrix detection *P* value to identify those transcripts that were reliably detected on the chip over background (*P* value ≤ 0.06). The reliably detected transcripts for each replicate were then filtered for differential expression that was significant from control (SLR ≥ 0.5 or ≤ -0.5). At this point, each replicate data set was represented by a different transcript list. These lists of reliably detected and differentially expressed transcripts were intersected to identify a common set of genes that passed the first 2 filtering steps in all replicates. Finally, transcripts common to all replicates were filtered on consistency of expression profile over time. Pearson's correlation coefficient (PCC) was used to identify transcripts with similar patterns of expression, and Euclidean distance (ED) was used to determine if a transcript's magnitude of expression over time was consistent. The PCC cutoff (PCC ≥ 0.7) was chosen based on statistical reasoning where any 2 profiles with a PCC greater than 0.7 are considered to be highly positively correlated, and the ED cutoff (ED ≤ 1.15) was chosen to be the median ED score across all replicate comparisons for WT and IFNAR^{-/-} data sets. In this work, an all-by-all comparison was done to obtain 3 PCC and 3 ED scores per gene, one per pair of replicates. Only 2 of the 3 scores for PCC and ED were required to pass the chosen cutoffs because requiring all 6 scores to pass was determined to be too strict (data not shown).

Consensus clustering and pattern identification

The WT and IFNAR^{-/-} filtered gene lists were clustered using the consensus clustering option in SC²ATmd version 2 (Olex and Fetrow 2011). A figure of merit (FOM) analysis (Yeung and others 2001) (available through SC²ATmd) was run on each data set to determine the number of clusters and clustering algorithm that created the most homogeneous clusters with respect to the ED metric. Based on the FOM results, each replicate was clustered twice using the K-means algorithm with ED as the clustering metric, which resulted in 10 starting clusters for WT and 8 clusters for IFNAR^{-/-}. SC²ATmd calculated the consensus over all 6 clustering runs, where genes found in the same cluster 100% of the time were identified as a consensus cluster.

While consensus clustering is able to identify those groups of genes that have high similarity in the expression

profile over time, it also breaks the data up into a large number of relatively small clusters—making the data difficult to analyze. Therefore, the average cluster profile was calculated and used to assign each cluster to a more general pattern of expression over time. The average gene expression for each time point was discretized into one of 3 values: UP, $SLR \geq 0.5$; DOWN, $SLR \leq -0.5$; or No Change, $-0.5 < SLR < 0.5$. Clusters having the same discretized pattern over time were assigned to the same *pattern* (Supplementary Fig. S2). Annotation analysis was run on each identified pattern as described below.

Annotation analysis

The Database for Visualization, Annotation, Integration and Discovery (DAVID) (Huang da and others 2009a, 2009b) was used to identify over-represented annotation terms in lists of differentially expressed genes, including WT and IFNAR^{-/-} pattern groups, common gene data sets, and others as described in Results. Each list was run through DAVID's Annotation Clustering Tool using the Affymetrix Mouse 430 2.0 chip as a background with all other settings left as default. Annotation groups were identified as significant if the Enrichment Score was greater than 1.3 (equivalent to a *P* value ≤ 0.05).

Integration of dynamic gene expression data with static network data

Integration of the kinetic gene expression data obtained here with known static protein interaction and metabolic networks was performed using the jActiveModules version 2.23 (JAM) plugin for Cytoscape (Ideker and others 2002; Smoot and others 2011). This network analysis was performed on the WT and IFNAR^{-/-} data sets independently as a separate analysis from the filtering and clustering described previously. Before performing a network analysis using JAM, a known protein interaction and reaction network was identified and gene expression data were converted for compatibility with JAM. In addition, a consensus approach to the original JAM algorithm (cJAM) was implemented to obtain active subnetworks that appeared a statistically significant number of times in multiple runs of JAM. Obtaining known interactions, processing of gene expression data, and the implementation of cJAM are described below.

Identifying known network interactions

The Cytoscape plugin BioNet Builder v2.0 (Avila-Campillo and others 2007) was used to query the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) in August of 2010 for all known mouse interactions and reactions present between any 2 genes represented on the Affymetrix Mouse 430 2.0 chip. The list of probe sets was imported into BioNet Builder, and all associated nodes (representing gene products) and edges (representing molecular interactions and reactions) for the *Mus musculus* genome were downloaded into Cytoscape version 2.8.1 (Smoot and others 2011) and visualized as a network. Due to the large scale of the complete *Mus musculus* network available through BioNet Builder, only those nodes that were represented on the microarray chip were downloaded. Self-edges and edges labeled as Shared

Compound were deleted from the network because neither was relevant to our work, where self-edges resulted in JAM returning single-node results, and Shared Compound edges were not related to physical protein interactions or metabolic pathways. The resulting mouse network contained 2,393 nodes, each uniquely identified by Entrez ID and represented on the microarray chip, connected by 32,342 edges. Interactions and reactions between gene products included 15,240 edges labeled as Enzyme-Compound interactions (EC), 440 edges labeled as Gene Expression interactions (GE), and 16,662 edges identified as Protein-Protein interactions (PP). JAM used this “global network,” as the background for identifying active subnetworks.

Data conversion and mapping to the global network

Gene expression data were converted for compatibility with JAM (Ideker and others 2002) using an in-house script written in MATLAB (MathWorks, Inc.). To identify those genes whose expression was significantly detected, the WT or IFNAR^{-/-} SLRs for each replicate and time point were independently filtered by detection *P* value, where all 3 time zero *P* values plus the respective experimental *P* value were required to be ≤ 0.06 . The mean SLR and standard deviation for each time point and replicate in the filtered lists were calculated. Next, the Affymetrix probe set IDs for each filtered replicate were mapped to the corresponding Entrez ID based on the Affymetrix annotations file downloaded from NetAffx (www.affymetrix.com). On occasion, several probe sets would map to a single Entrez ID, or one probe set would map to several Entrez IDs creating many-to-one or a one-to-many relationship, respectively. While a one-to-many relationship was considered trivial, having several probe sets mapping to a single Entrez ID was a problem because only one SLR value could be represented in the network per Entrez ID. To resolve this issue a “summary SLR” was calculated as the average SLR value across all probe sets mapping to the same Entrez ID. Finally, the SLR and summary SLR values were converted to the exclusive range of (0,1) by integrating the cumulative distribution function (Equation 1) (Abramowitz and Stegun 1972) over each replicate for each time point (a normal distribution was assumed). Integration was done relative to the respective mean and standard deviations for each replicate and time point for a given gene's SLR value. SLR values represented a z-score on the distribution where in Equation 1, Φ represents the fraction of SLR values below a given gene's SLR, and Equation 2 yields the fraction of genes with a greater absolute z-score compared with a given gene's SLR for the given replicate and time point.

$$\Phi(x_{ij}, \mu_i, \sigma_i) = \frac{1}{\sigma_i \sqrt{2\pi}} \int_{-\infty}^{x_{ij}} \exp\left(-\frac{(x_{ij} - \mu_i)^2}{2\sigma_i^2}\right) dx \quad \text{Eq 1.}$$

$$\text{converted value} = 1 - |\Phi(x_{ij}, \mu_i, \sigma_i) - \Phi(-x_{ij}, \mu_i, \sigma_i)| \quad \text{Eq 2.}$$

In Equations 1 and 2, μ and σ are the mean and standard deviation, respectively, of all *P* value-filtered SLR values for each replicate *i*, and *x* is the SLR value for gene *j* under replicate *i*.

Once array data were converted into a range useable by JAM, data were mapped onto the global KEGG network using the Entrez IDs. Because of the P value filter, not all genes represented in the network contained converted expression values for every time point and replicate. Thus, missing data were given a converted value of 0.5, providing that gene with a 50% chance of appearing in subnetwork results for a given replicate and time point.

Consensus *jActiveModules* analysis

Network analysis was performed using the described global mouse network and converted WT and IFNAR^{-/-} gene expression data. Details of the original JAM algorithm have been described previously (Ideker and others 2002; Chuang and others 2007). Briefly, converted gene expression data are overlaid onto the known network, and an annealing search algorithm with a random initial start is used to search the network for *active subnetworks*. Subnetwork space is searched by adding or removing nodes one at a time from the current selection. Subnetworks are scored by JAM using the average of the selected z-scores. Once subnetwork scores no longer significantly improve, the search is halted and the current subnetwork selection is deemed as active (active subnetworks). A known issue with this approach is that it is impossible to search the entire subnetwork space (Ideker and others 2002), therefore, only part of the network space is searched with random initialization. The stochastic nature of this approach results in different subnetworks with each run; therefore, a consensus approach was implemented to identify those subnetwork nodes that appeared as active a significant number of times over multiple runs of JAM.

Consensus JAM (cJAM) was performed on the WT and IFNAR^{-/-} time courses independently as shown in Supplementary Fig. S3. The SLRs of each replicate data set were overlaid onto the global network individually, and a JAM search was run 10 times, using a different starting seed each time. Subnetwork results from each of the 10 runs were filtered to remove those with a score less than 3.0 (Cline and others 2007). The remaining subnetworks were analyzed using an in-house script that calculated the probability of each node appearing in the search results a given number of times. Statistical significance for each node returned by cJAM was calculated as follows. Assume that $G_i = \{V_i, E_i\}$ represents the collection of graphs (G), made up of a set of nodes (vertices V) and edges (E), returned from the i th of k total runs of JAM over the global network with t total nodes. Let max be the number of nodes in the largest node set V_i returned by JAM for any one run. Let U be the union of all node sets, thereby defining the set of nodes seen across any run of JAM with its size represented as $|U|$. For each node u in the union U , a count of how many node sets the node appeared in was recorded. High values for this count would reveal if a node was seen in many of the repeated searches. To quantify how often a node should appear to be statistically significant, the following process is used. Assume that max/t , the maximum number of nodes returned from any run divided by the total number of nodes searched in the *global network*, represents the probability of a node being selected by chance. This value corresponds to the probability of a node being selected under the most “aggressive” searcher. Given this, the probability of a node being selected was computed using the binomial distribution, with parameters of k trials and

probability, max/t , of success within a trial. The nodes in U can then be rank ordered by this probability to identify those that are statistically significant. Since hundreds or thousands of nodes are being evaluated under the binomial distribution, a Bonferroni correction for repeated tests is used. To apply the Bonferroni correction, the cutoff probability for statistical significance is divided by the number of repeated tests being used, which in this scenario is $|U|$, the number of nodes seen across all runs of the search algorithm.

Once the set of nodes that appeared a statistically significant number of times was determined, edges connecting those nodes were mined from the global network to create the consensus subnetwork. This was done for each replicate and each time point. The consensus subnetworks for each replicate were then intersected to obtain one subnetwork for each time point. Finally, the subnetworks for each time point were unioned to generate an active subnetwork for WT and an active subnetwork for IFNAR^{-/-}, where any given node was deemed active in all replicates for at least one time point.

Results

DC maturation and cytokine production induced by Poly I:C are compromised in cells lacking the type I interferon receptor

Poly I:C is known to cause a strong DC maturation response and has been used as an adjuvant in several vaccine preparations (Mian and others 2013). To assess the impact of the type I interferon response on the DC maturation phenotype induced by Poly I:C, we treated WT or IFNAR^{-/-} GM-CSF-derived murine bone marrow DCs with Poly I:C for 24 h. The expression of cell surface costimulatory molecules and secreted cytokines was then measured by flow cytometric analysis and ELISA assay (Fig. 1). CD40, CD86, and H-2K^b were all significantly upregulated over untreated background upon Poly I:C treatment of WT DC. However, in IFNAR^{-/-} DC, the increase in CD40 and CD86 expression was reduced compared to WT and the expression of H-2K^b was not significantly changed over background (Fig. 1A). CD80 expression was not significantly upregulated by Poly I:C in either WT or IFNAR^{-/-} DC.

We next measured the expression of inflammatory cytokines by WT and IFNAR^{-/-} DC upon Poly I:C treatment (Fig. 1B). Poly I:C induced the expression of IL-6 and IFN- β , but not IL-12p40 or TNF- α . The expression of IL-6 was ablated in IFNAR^{-/-} DC, and IFN- β expression was reduced by over 50% compared to WT DC.

T-cell priming capacity of IFNAR^{-/-} DC is reduced compared to WT

We next wanted to determine how these differences in costimulatory activity and MHC expression would impact the T-cell priming capacity of these cells and their ability to present antigen to CD8 T cells. To determine if IFNAR mediated the ability of DC to present exogenous antigen, DCs from WT and IFNAR^{-/-} mice were loaded with OVA peptide (1 ng/mL) and treated with Poly I:C (or no treatment) for 24 h. CFSE-stained, naive OT-1 T cells were then added to the culture to detect the presentation of the OVA peptide. The T cells were harvested on day 3 of culture and assessed by flow cytometry for proliferation (Fig. 2). In the absence of treatment, neither WT nor IFNAR^{-/-} DC induced

FIG. 1. Impact of IFNAR on costimulatory molecule and cytokine expression by DC. DCs were generated from WT and IFNAR^{-/-} mice and treated with Poly I:C. Twenty four hours postinfection, supernatants were collected for cytokine analysis by ELISA and surface expression of costimulatory molecules was assessed by flow cytometry. (A) Expression of CD40, CD80, CD86, and H-2K^b following treatment is shown as a fold increase over untreated background. (B) IL-12p40, IL-6, TNF-α, and IFN-β upon Poly I:C stimulation were determined by ELISA. Cytokine production by untreated DC was negligible. Results are compiled from 3 to 5 independent experiments. *Indicates *P* value < 0.05, ***P* value < 0.01, ****P* value < 0.005. DC, dendritic cell; WT, wild type.

a strong T-cell proliferation response (Fig. 2A). Poly I:C treatment increased T-cell proliferation significantly by enhancing division in both WT and IFNAR^{-/-} populations. However, the IFNAR^{-/-} DC still induced a lower frequency of T cells to enter division (Fig. 2B).

To next examine the role of IFNAR in the processing and presentation of antigen encoded by a bacterial pathogen, we infected WT and IFNAR^{-/-} DC with recombinant *Listeria monocytogenes* expressing the OVA protein (*Lm-OVA*), stopped the infection with antibiotics after 2 h, and then measured the response of OT-1 T cells 24 h later (Fig. 3). Compiled data from several experiments showed that WT DC stimulated strong proliferation and IFN-γ production by OT-1 T cells when infected with 1 or 3 *Lm* per cell (Fig. 3A,

B). However, IFNAR^{-/-} DCs were less able to stimulate T-cell division (Fig. 3B), and the magnitude of IFN-γ production was much lower in T cells primed by IFNAR^{-/-} DC (Fig. 3A). To gain a more comprehensive view of the contribution of type I interferon signaling to DC maturation induced by Poly I:C, we next performed global gene expression analysis of this process using microarrays.

Fewer transcripts are significantly changed in IFNAR^{-/-} DC compared to WT in response to poly I:C treatment

WT and IFNAR^{-/-} DCs were treated with Poly I:C for 0 (control), 1, 2, 3, 6, 9, 12, 18, or 24 h. RNA was then

FIG. 2. Antigen-presenting capacity of IFNAR^{-/-} DC. WT and IFNAR^{-/-} DCs were treated with Poly I:C and pulsed with OVA peptide (1 ng/mL) for 24 h. CFSE-stained naive OT-1 T cells were added to the culture and allowed to respond for 72 h. The degree of T-cell proliferation was then measured by flow cytometry by dilution of CFSE. (A) Histograms of proliferation. (B) Compiled proliferation data from 5 independent experiments. *Indicates *P* value < 0.05.

FIG. 3. Antigen processing, presentation, and T-cell activating capacity of DC lacking IFNAR. WT and IFNAR^{-/-} DCs were infected with the indicated multiplicity of infection (MOI) of *Lm*-OVA for 24 h and then CFSE-labeled OT-I TCR transgenic T cells were added to the cultures. Seventy two hours later, the T cells were restimulated with OVA peptide in the presence of GolgiPlug for 5 h, and the production of IFN-γ was assessed by intracellular cytokine staining. (A) Magnitude of IFN-γ production as measured by mean fluorescence intensity of CD8⁺ T cells. Data were compiled from 3 independent experiments. (B) Division index calculated by FlowJo after priming by the indicated DC infected with increasing MOI of *Lm*-OVA. *Indicates *P* value < 0.05, ** indicates *P* value < 0.005, *** indicates *P* value < 0.001, **** indicates *P* value < 0.0005.

extracted, and microarray analysis was carried out on each sample. Analysis and filtering of the microarray data revealed that 2,709 transcripts were significantly differentially expressed in WT DC at some point during the time course, while only 1,274 transcripts fit this criterion for IFNAR^{-/-} DC (Supplementary Tables S1 and S2, respectively). Comparison of filtering results (Table 1) revealed that the SLR filter removed, on average, 30% more transcripts from the IFNAR^{-/-} data than from WT, whereas the other filtering steps reduced the number of transcripts in WT and IFNAR^{-/-} by a similar percentage. The larger transcript reduction caused by the SLR filter indicates that the IFNAR^{-/-} response had, in general, fewer genes that responded significantly (>1.4-fold) to poly I:C treatment. Results that follow show that this reduction is not just a reduction in the number of genes that respond to poly I:C, but is a reduction in the overall magnitude of response across all genes.

Two prominent gene annotations that were unique to the WT DC response, but failed to pass our filtering strategy, were interferon beta and CD86. The interferon beta gene failed to pass the *P* value cutoff in one replicate—so was not included in the final data set. However, the pattern of its expression in WT DC reflects 2 phases: an early upregulation and a second phase in which its expression is amplified

(Supplementary Fig. S5). These results are consistent with the 2 phases of interferon response, induction and signaling (Matsumoto and Seya 2008; Bonjardim and others 2009). As predicted, the second phase of IFN-β expression was not observed in the IFNAR^{-/-} cells, which is consistent with the knowledge that, without IFNAR, they are unable to receive or transmit type I interferon signals. Likewise, while CD86 was strongly upregulated in WT DC, it was actually downregulated in IFNAR^{-/-} DC. This gene failed to pass the consistency filter (Supplementary Fig. S4). This stringent filtering strategy enables us to focus on prominent and novel gene expression patterns with a high degree of confidence.

Response to poly I:C by IFNAR^{-/-} DCs lacks the prominent downregulated program observed in WT

To globally compare the poly I:C response of WT and IFNAR^{-/-} DC at the transcriptional level, the gene expression distributions for both filtered data sets (2,709 and 1,274 transcripts, respectively) were compared (Fig. 4A). In the early time points (1, 2, and 3 h post-treatment), a similar distribution of gene expression in both cell types was observed with most genes changing little (expression is

TABLE 1. NUMBER OF TRANSCRIPTS REMAINING FOR WILD-TYPE AND IFNAR^{-/-} DATA SETS FOLLOWING EACH DIFFERENTIAL EXPRESSION FILTERING STEP

Replicate	Total transcripts	Detection P value filter	SLR filter	Overlap analysis	PCC And ED filter
WT Rep1	45,101	16,602	10,922	6,715	2,709
WT Rep2	45,101	16,155	10,326		
WT Rep3	45,101	15,800	9,943		
IFNAR Rep1	45,101	17,787	5,530	2,920	1,274
IFNAR Rep2	45,101	16,438	6,574		
IFNAR Rep3	45,101	17,866	5,413		

WT, wild type; PCC, Pearson's correlation coefficient; ED, Euclidean distance; SLR, signal log ratio.

FIG. 4. SLR distributions reveal IFNAR^{-/-} DCs lack the downregulated response present in WT; however, a similar upregulated response is observed at later time points. (A) The distribution of differential gene expression data for WT and IFNAR^{-/-} DC at each time point after poly I:C treatment is plotted. *Blue shades* are WT replicates, which contain 2,709 probe sets each. *Red shades* are IFNAR^{-/-} replicates, which contain 1,274 probe sets each. (B) Transcripts that were significantly upregulated at the 24-h time point (gray box in A) in WT and IFNAR^{-/-} were compared using the official gene symbols. Only 79 unique gene symbols were present in both data sets. Significant annotations [identified using DAVID; <http://david.abcc.ncifcrf.gov> (Huang da and others 2009a, 2009b)] for each gene subset are listed in the corresponding sections of the Venn diagram. SLR, signal log ratio.

normally distributed around an SLR of 0). However, around 6 h a shift in WT gene expression occurred, with the emergence of a large number of downregulated genes and a smaller peak of upregulated genes (blue graph, Fig. 4A). This bimodal distribution became more prominent at 9 h and remained strong through 24 h. The gene expression distribution in the IFNAR^{-/-} DC did not change until 9 h when an upregulated response became evident (red graph, Fig. 4A). Interestingly, at 12, 18, and 24 h, a similar number of IFNAR^{-/-} and WT genes were upregulated, but the downregulated response was largely not observed in the IFNAR^{-/-} cells (Fig. 4A). The major function annotations associated with the downregulated response in the WT cells were several associated with RNA—its processing, binding, transport, catabolism, and so on, as well as ribosome biogenesis, translation, and various nucleolar structures (Table 2). These annotations were much less prominent in the IFNAR^{-/-} cells (Table 3).

The genes upregulated in DC lacking IFNAR are distinct from those upregulated in WT DC

While the downregulated response to poly I:C was much less prominent in IFNAR^{-/-} cells than WT, a similar number of genes were upregulated in both cell types at 18 and 24 h (611 in WT, 609 in IFNAR^{-/-} at 24 h,

Supplementary Tables S1 and S2, respectively). It was therefore reasonable to ask if the same genes were upregulated in both DC types. Intersecting the upregulated genes at 24 h post-treatment in WT and IFNAR^{-/-} DC revealed that only 79 genes were shared between the 2 responses (Fig. 4B), indicating that most of the genes involved in these responses were distinct.

We next queried the functions associated with these upregulated genes. The genes upregulated in WT DC at 24 h exhibited significant function annotations, which included intracellular transport, proteolysis, programmed cell death, ubiquitination, lysosomal function, and regulation of interferon and cytokine production (Supplementary Table S3). The major function annotations associated with the 24-h upregulated genes in IFNAR^{-/-} DC included a distinct and smaller set of lysosome-associated genes (Supplementary Table S4). The functions of carbohydrate and lipid metabolism, as well as oxidative stress, were annotations unique to the IFNAR^{-/-} DC. The 79 upregulated genes common to WT and IFNAR^{-/-} DC exhibited functions, including lysosomal function and the ubiquitin conjugation pathway (Supplementary Table S5). The annotation of programmed cell death-associated genes was also shared between IFNAR^{-/-} and WT DC; however, this annotation was found significant based on distinct subsets of genes, thus, the

TABLE 2. SIGNIFICANT DAVID ANNOTATIONS FOR WILD-TYPE PATTERNS HAVING AT LEAST 10 MEMBERS

Pattern number	Total gene count	1 h	2 h	3 h	6 h	9 h	12 h	18 h	24 h	Overrepresented annotations
5	121			D	D	D	D	D	D	RNA processing, ribosome biogenesis, RNA binding, Cajal body, methyltransferase, polyamine metabolic process, mRNA processing, chromatin/chromosome organization
8	508				D	D	D	D	D	Nuclear lumen, ribosome biogenesis, RNA processing, spliceosome, translation, KEGG: Ribosome, RNA recognition motif, mitochondrion, exosome, translational initiation, single-stranded RNA binding, methyltransferase activity, WD40 repeat, RNA transport, protein import, protein complex biogenesis, nuclear transport, DNA-directed RNA polymerase, helicase activity, mitochondrion organization, RNA catabolic process
18	31				D	D	D	D		Oxidation reduction, mitochondrion, transit peptide
15	32				D	D	D			None (mitochondrion)
9	496					D	D	D	D	Ribonucleoprotein complex, ribosome, RNA processing, spliceosome, nuclear lumen, mitochondrion, oxidation reduction, unfolded protein binding, Chaperone, RNA recognition motif, translational initiation, RNA transport, WD40 repeat, helicase activity, DEAD box, protein transport, regulation of translation, ribosome biogenesis, ATP binding
6	82					D	D	D		Organelle lumen, mitochondrial ribosome, mitochondrial matrix
7	38					D	D			Mitochondrion, ribosomal protein, transit peptide
11	221						D	D	D	Ribosome, translation, RNA binding, spliceosome, RNA recognition motif, mitochondrial part, respiratory chain, NADH dehydrogenase activity, oxidoreductase, RNA localization, KEGG: Oxidative phosphorylation, generation of precursor metabolites and energy, ribosome biogenesis
14	145							D	D	RNA splicing, ribosome, cytoskeleton, RNA binding, RNA localization, nuclear lumen, Ubiquitin associated, RNA splicing
19	24							D		None (cell cycle)
16	17								D	KEGG: Renal-cell carcinoma, KEGG: Pathways in cancer
10	67			U	U	U	U	U	U	Protein kinase cascade, NF-kappaB cascade
12	82				U	U	U	U	U	Lysosome, lytic vacuole, peptidase activity, zymogen, proteolysis
3	105				U	U	U	U		Cellular macromolecule catabolic process, proteolysis, endomembrane system
2	158					U	U	U	U	Zinc finger, protein transport, protein localization, endosome, Golgi apparatus, transmembrane region, lysosome, lytic vacuole, GTPase activity, GTP binding, purine nucleotide binding
13	21					U	U	U		None (proteolysis)
4	55						U	U	U	Integral to organelle membrane, endoplasmic reticulum
17	13						U			Serine/threonine protein kinase, protein kinase activity, ATP binding, phosphorus metabolic process
1	222							U	U	Protein catabolic process, microtubule, cytoskeleton, motor activity, protein localization, Golgi apparatus, negative regulation of cellular component biogenesis, actin binding, ligase activity

apoptosis annotation does not appear in the 79 genes from the intersection of these data sets.

WT and IFNAR^{-/-} cells have 254 differentially expressed genes in common

Intersection of the 2,709 WT and 1,247 IFNAR^{-/-} differentially expressed data sets identified 254 transcripts that were differentially expressed in response to poly I:C in both cell types (Supplementary Fig. S5A). Comparison of gene

expression patterns between WT and IFNAR^{-/-} DC in the 254 overlapping genes reveals that the temporal profile was similar in most genes, but that the scale was different. To compare the WT and IFNAR^{-/-} responses among these 254 probe sets, ED, which compares magnitude of 2 time profiles, and PCC, which compares pattern of 2 time profiles, were calculated for each of the 254 common genes. The ED versus PCC scores for each gene were plotted in a scatter plot (Supplementary Fig. S5B) and divided into 4 groups using hierarchical clustering, where group 1 consists of those genes

TABLE 3. SIGNIFICANT DAVID ANNOTATIONS FOR IFNAR^{-/-} PATTERNS HAVING AT LEAST 10 MEMBERS

Pattern number	Total gene count	1 h	2 h	3 h	6 h	9 h	12 h	18 h	24 h	DAVID annotations
14	13				D	D	D	D	D	None (acetylation)
11	29					D	D	D	D	DNA replication, cell cycle process
2	153						D	D	D	Mitotic cell cycle, cell division, M phase, chromosome, kinetochore, RNA processing, RNA binding, nuclear lumen, DNA packaging, DNA replication, KEGG: Cell Cycle, nucleotide binding, ATP binding, microtubule, kinesin, rRNA processing, heterochromatin, chromatic binding, histone methylation, RNA localization, chromosomal protein, response to DNA damage stimulus, DNA repair, sex chromatin, chromatin organization, negative regulation of transcription
6	138							D	D	Mitosis, cell division, M phase, cell cycle, RNA binding, methylation, chromosome, kinetochore, spliceosome, nucleolus, mRNA transport, RNA localization, protein transport
10	65								D	Nuclear lumen, RNA recognition motif, RNA splicing, spliceosome, nucleolus, isopeptide bond
1	63		U	U						Cellular response to stress, response to DNA damage stimulus, DNA repair
13	10		U							None
3	41				U	U	U	U	U	None (negative regulation of biosynthetic process)
5	93					U	U	U	U	Transmembrane region, integral to membrane, hydrogen peroxide metabolic process, oxidoreductase, glycoprotein
8	78						U	U	U	None (ATP metabolic process)
12	11						U			None (plasma membrane part)
9	181							U	U	Lytic vacuole, lysosome, carbohydrate catabolic process, oxidoreductase, glucose metabolic process, energy reserve metabolic process
4	167								U	Lysosome organization, lytic vacuole, skeletal system development, tetratricopeptide repeat, carbohydrate metabolic process, oxidoreductase activity, fatty acid biosynthetic process, coenzyme catabolic process

with most similar expression profiles in both WT and IFNAR^{-/-} (Fig. 5A, B and Supplementary Fig. S5C) and group 4 contains those genes that had the most diverse response between WT and IFNAR^{-/-} (Fig. 5G and Supplementary Fig. S5F).

Two hundred thirty of 254 transcripts exhibited similar temporal response patterns to poly I:C treatment in both cell types (group 1, Fig. 5A, B). These genes were separated into those transcripts that were upregulated in WT over the 24-h period (Fig. 5A) and those that were downregulated (Fig. 5B). Annotation analysis of the downregulated genes identified an enrichment of functions involved in RNA processing such as binding, acetylation, and methylation. There were no DAVID-identified function annotations associated with the group 1 upregulated genes that met our *P* value requirements for significance. However, among the upregulated genes were several known to be involved in lysosomal transport and function (catabolic processing).

The transcripts in groups 2, 3, and 4 represent genes that were significantly changed in both WT and IFNAR^{-/-} DC, but whose patterns of expression were either poorly correlated (group 2; Fig. 5C, D) or inversely correlated (group 3, Fig. 5E, F and group 4, Fig. 5F). The 13 genes in group 2 varied widely in ED scores and had a narrow but low range of PCC scores (between roughly 0 and 0.4). Thus, expression of these genes was not strongly correlated between WT and IFNAR^{-/-}. The magnitude of change in WT genes for

this set was generally more extreme (positive or negative) than in IFNAR^{-/-} (Fig. 5C, D). Among the notable transcripts in this group were MyD88 and the IL-1 receptor antagonist, both of which are key mediators of inflammation. MyD88, an adaptor protein involved in TLR- and IL-1R/IL-18R-mediated signaling (Adachi and others 1998; Kawai and others 1999), exhibited an opposite pattern of expression in IFNAR^{-/-} compared to WT. In WT it was upregulated, but in IFNAR^{-/-} it was downregulated. The IL-1 receptor antagonist (IL-1rn), which negatively regulates IL-1 signaling and inflammation (Liao and others 1984, 1985), was strongly upregulated in WT DC, but generally unchanged to weakly upregulated in IFNAR^{-/-} DC. In addition, several genes involved in lysosomes, vacuoles, and intracellular traffic appear in group 2. Sorting nexin 10 (Snx10) is involved in endosomal homeostasis (Qin and others 2006), Acp2 is a lysosomal phosphatase (Jones and Kao 1978; Haggren and Kolodrubetz 1988; Bailey and others 2013), and Pik3R6 regulates the activity of Pik3, which is known to participate in mast cell degranulation (Suire and others 2005).

Group 3 included only 8 transcripts, with a narrow ED range, yet a large PCC range from 0 to -0.7. The notable feature of this group is that they had inverse expression profiles in WT versus IFNAR^{-/-} DC (Fig. 5E, F). Interestingly, 6 of the 8 transcripts in this group were downregulated in WT DC (Fig. 5F, black/gray lines), yet

FIG. 5. WT and IFNAR^{-/-} DCs regulate a common set of transcripts that have similar and inverse responses to poly I:C. The intersection of WT and IFNAR^{-/-} filtered data sets identified 254 differentially expressed transcripts in both cell types in response to poly I:C treatment. These genes were divided into 4 groups based on whether or not they had similar expression profiles in WT and IFNAR^{-/-} using PCC and ED scores (Supplementary Fig. S5). (A, B) Group 1 genes that were up- (A) or downregulated (B) in WT had similar responses in WT (black) and IFNAR^{-/-} (red) over time (average PCC=0.79, average ED=2.28). (C, D) Group 2 genes (up- (C) and downregulated (D) in WT) had little correlation between WT and IFNAR^{-/-} profiles (average PCC=0.19, average ED=3.40). (E, F) Group 3 genes [up- (E) and downregulated (F) in WT] had a slight inverse correlation between WT and IFNAR^{-/-} profiles (average PCC=-0.32, average ED=4.34). (G) Group 4 genes (all upregulated in WT) had a slight inverse correlation between WT and IFNAR^{-/-} profiles and a large difference in magnitude of expression (average PCC=-0.40, average ED=9.89). PCC, Pearson's correlation coefficient; ED, Euclidean distance.

upregulated in IFNAR^{-/-} DC (Fig. 5F, red/pink lines). Included in this functionally diverse group of genes was a cytoskeletal protein (Myo5a), a translation initiation factor (Eif4B), and a regulator of insulin signaling (Scd1).

Finally, the last group of transcripts (group 4, Fig. 5F) contained only 3 probe sets, TREX1, Tor1Aip1, and a predicted gene, 9530028C05. The expression of these genes is the most different between WT and IFNAR^{-/-}, with negative PCC scores and very high ED scores. The WT cells highly upregulated these genes (Fig. 5F, black/gray lines), while in the IFNAR^{-/-} the change was modest (Fig. 5F, red/pink lines), and the small change observed was opposite that of WT. The most notable of these was the exonuclease, TREX1, which was strongly upregulated in WT cells, es-

pecially at later time points. However, in IFNAR^{-/-}, TREX1 was only modestly upregulated early followed later by downregulation. TREX1 has previously been identified as an IFNAR-dependent gene involved in cell death induced by granzyme A, as well as having antiviral functions (Crow and others 2006; Lee-Kirsch and others 2007; Hasan and others 2013).

Clustering identifies various patterns of response to poly I:C, with IFNAR^{-/-} DC exhibiting a delayed and different response from WT

To identify those genes with similar response patterns to poly I:C, the lists of differentially expressed genes identified

in WT and IFNAR^{-/-} DC (2,709 and 1,274 transcripts, respectively) were clustered using SC²ATmd's consensus clustering option (Olex and Fetrow 2011) and clusters were then assigned to more general patterns of expression (see Materials and Methods section). In WT, 2,495 transcripts were assigned to 34 patterns (a summary of patterns with at least 10 members is provided in Table 2) with 214 identified as outliers by consensus clustering. Outlier genes did not consistently cluster with any other gene. Likewise, in IFNAR^{-/-}, 1,157 transcripts were assigned to patterns (a summary of patterns with at least 10 members is provided in Table 3) with 117 identified as outliers. In WT and IFNAR^{-/-}, we observed up- and downregulated expression patterns starting either early or later in the process (Tables 2 and 3, respectively). Interestingly, the patterns indicate that the majority of gene expression changes were sustained until the end of the time course, with few being transient and ending before 24 h.

In WT DC, the downregulated response was the most prominent, as previously reported (Olex and others 2010), with 70% of genes assigned to a downregulated pattern at early, middle, and late time points throughout the 24-h period (Table 2). Most of the patterns of downregulation were sustained through the end of the time course once induced, with the exception of 4 transient patterns in which downregulation ended before 24 h (patterns 6, 7, 15, and 18). The function annotations associated with the sustained downregulated patterns were largely associated with ribosomes, RNA processing, and translation. Interestingly, the more transient downregulation patterns were primarily associated with mitochondria, oxidation, and reduction.

Genes associated with the upregulated expression patterns in the WT DC displayed a very different functional profile than the downregulated WT response. At the earliest time points, the protein kinase and NF- κ B cascades were upregulated followed closely by function annotations such as lysosome, lytic vacuole, and proteolysis. These findings are consistent with several reports showing activation of the NF- κ B signaling pathway upon Poly I:C triggering of TLR3 (Alexopoulou and others 2001; Gelman and others 2004; Gautier and others 2005). A cohort of genes involved in protein transport and localization were upregulated in a sustained manner starting at 9 h, followed by a large number of genes involved in protein localization and cytoskeletal motor activity, later in the process at 18 h. In general, the upregulated response in WT cells reflects quite closely the hallmark changes associated with the DC maturation inflammatory response, protein degradation, and migration as was observed in our previous study (Olex and others 2010).

The downregulated expression patterns observed in the IFNAR^{-/-} DC (Table 3) contained markedly fewer genes than in WT (Table 2)—398 compared to 1,691 transcripts from the largest 10 patterns of IFNAR^{-/-} and WT, respectively. We also observed that the downregulated response was delayed kinetically in IFNAR^{-/-} DC, compared to WT, with most genes not changing significantly until 12 h or later. The function annotations associated with the IFNAR^{-/-} DC downregulated patterns were generally associated with cell cycle—DNA replication, mitosis, cell division, DNA repair, and chromatin organization. This is in contrast to the WT DC in which the predominant functions in the downregulated group included RNA processing, ribosomes, and translation. Thus, while the WT cells appear

to shut down RNA processing and protein translation, IFNAR^{-/-} DC shut down cell cycle and DNA replication.

Unlike WT DC, the IFNAR^{-/-} response to poly I:C is dominated by upregulation: almost 63% of genes were assigned to a pattern that was upregulated at some point in the process. This upregulated response is similar in magnitude to the WT response at later time points—609 genes upregulated in WT versus 611 in IFNAR^{-/-} at 24 h (Fig. 4B)—and does include some similar function annotations such as the lysosome and lytic vacuole. However, the IFNAR^{-/-} upregulated response also has several distinct features from WT. For example, IFNAR^{-/-} DCs have 2 patterns (patterns 1 and 13, Table 3) that are transiently upregulated early between 2 and 3 h and include genes involved in DNA damage repair, patterns unique to these cells. In addition, as observed in the IFNAR^{-/-} downregulated response, the majority of the genes were delayed kinetically compared to WT, with most changing at 18 and 24 h. Genes associated with catabolic processes are found in the WT and IFNAR^{-/-} DC; however, the targets of catabolism seemed to be predominantly carbohydrates and lipids for the IFNAR^{-/-} DC in contrast to proteins, as observed in WT.

Network analysis reveals known and novel pathways affected by poly I:C treatment unique to WT or IFNAR^{-/-} cells

The integration of static protein interaction or other network data with dynamic understanding of systems is the subject of much research [reviewed in (Pantel and others 2014)]. The time-based gene expression profile, which we collected here, provides a unique opportunity to integrate static network data with this dynamic experimental data, to identify subnetworks active in WT and IFNAR^{-/-} DC following poly I:C treatment. The WT and IFNAR^{-/-} data sets were subjected to a network analysis using our consensus version of the Cytoscape plugin JAM (Ideker and others 2002; Smoot and others 2011). This analysis identified active subnetworks for WT and IFNAR^{-/-} (Fig. 6) that included expected differences as well as novel pathways not previously reported to be associated with DC maturation.

Among the more predictable subnetworks was a large group of cytokine signaling regulators linked by protein-protein interaction and transcriptional regulation that were active over the WT DC time course (Fig. 6A, arrow 1). There was an especially large cluster enriched in cytokine receptors whose transcripts were generally upregulated: IFN- γ R2, IL-15Ra, IL-2R β , IL-7R, IL-13Ra, Crf2- TSLPR, as well as the IL-6 receptor that was downregulated (Fig. 6A). In addition, *STAT1* and *STAT2*, known to be involved in interferon-mediated signaling and transcriptional regulation, had upregulated transcripts, and several proteins downstream of these, including *CXCL10*, *CD40*, *CD86*, were also strongly upregulated transcripts. Interestingly, several Suppressor of Cytokine Signaling (*Socs3*, *Socs5*, *Socs7*) transcripts were downregulated in WT DC (Fig. 6A, arrow 1). Strikingly, the majority of nodes found in this network were absent in the IFNAR^{-/-} DC (Fig. 6B, gray nodes/edges).

While many of the STAT1- and STAT2-dependent cytokine signaling responses were absent in the IFNAR^{-/-} DC, these DCs exhibited a unique subnetwork of NF- κ B signaling molecules and transcriptional regulators (Fig. 6B, arrow 2). This subnetwork was upregulated early in the

FIG. 6. Active subnetworks identified by jActiveModules consensus for WT and IFNAR^{-/-} reveal many similarities and differences. A cJAM analysis was run on WT (**A**) and IFNAR^{-/-} (**B**) gene expression data. WT and IFNAR^{-/-} results were unioned for comparison purposes only. Colored nodes and edges highlight cJAM results for the respective cell type (**A**, Wild Type; **B**, IFNAR^{-/-}), and the shadow network (gray) indicates subnetworks that were active in the other cell type. Edges are formatted according to KEGG definitions (Kanehisa and Goto 2000): orange, protein-protein; green, enzyme-compound; blue, gene expression. Nodes are colored based on the average 24-h summary SLR for WT (**A**) and IFNAR^{-/-} (**B**), where red indicates upregulation, blue indicates downregulation, and white indicates no change from control. Numbered arrows indicate areas of interest that are discussed in the main text.

process (2–6 h) followed by downregulation at later time points and was not observed in WT DC. The notable members of this pathway included Rel α , Rel β , Traf1, Traf3, Traf4, Traf5, Nf κ b2, Nf κ b1b, Nf κ id, Nf κ ie, Traf6, and Map3k8.

Another prominent subnetwork observed in WT DC included a large group of genes involved in antigen processing and presentation and MHC class I (Fig. 6A, arrow 3). We anticipated that these might be absent in the IFNAR^{-/-} DC because IFNAR is crucial for MHC class I expression (Langer and others 1992; Cheng and others 2004; Pogue and others 2004; Moro and others 2011). Among the nodes found in the WT subnetwork at 24 h were several classical and nonclassical MHC molecules (H-2D1, H-2K1, H-2T9, H-2T10, H-2T22, H-2T23, H-2T24, H-2Q7, and H-2 M3), peptide loading chaperones (H-2Oa, Tapbp), peptide transporters (Tap1, Tap2), and Fc receptors (Fcgr1, Fcgr4). All of these genes were upregulated beginning around 3 h in WT DC and were generally unchanged or slightly downregulated in IFNAR^{-/-} throughout the time course (Fig. 7). These findings support our T-cell priming data indicating the IFNAR^{-/-} DC are compromised in their ability to process and present antigen through MHC class I to CD8⁺ T cells (Figs. 2 and 3).

In the IFNAR^{-/-} DC, we did find an active subnetwork at 24 h related to antigen processing and presentation, but the nodes in this network were almost completely distinct from those in the WT subnetwork (Fig. 6B, arrow 4). Only one

MHC molecule was shared with WT, H-2T23, which was modestly upregulated. The chaperone molecules H2-DMa, H2-DMb1, and H2-DMb2 were unique to the IFNAR^{-/-} DC and were downregulated. Given the function of these molecules in peptide loading into MHC, these data further suggest that these cells are likely compromised in presentation of antigen by MHC class II. Intriguingly, a unique family of cathepsin transcripts was also observed in the IFNAR^{-/-} DC antigen processing subnetwork, including Ctsb, Ctst, and Ctss, which was all modestly upregulated. This may suggest that the MHC class II processing pathway remains active or partially active in the absence of IFNAR. Further experiments will be required to address this possibility.

This analysis identified a novel active subnetwork in WT DC that included a large group of gene products involved in nucleotide synthesis, metabolism, and DNA repair (Fig. 6A, arrow 5). Nt5c3, Pde7b, Xdh, Pnp1, and Pnp2 were among the proteins whose transcripts were strongly upregulated genes in this subnetwork, while the transcripts of Rrm2, Dut, Tys, Atic, Uck2, Impdh2, and Cad were strongly downregulated. This subnetwork was almost completely unique to WT DC.

Interestingly, a subnetwork associated with redox activity was observed to be active in the IFNAR^{-/-} DC, but not in WT, consistent with our observation of oxidative stress response in the analysis of function annotations for the upregulated genes in IFNAR^{-/-} (Table 3). A large number of transcripts of proteins involved in glutathione synthesis and

FIG. 7. Gene expression dynamics of MHC/immune response subnetwork identified by cJAM. The gene expression levels for each node/gene in the network from each time point are overlaid onto the union of cJAM-identified subnetworks that contains various MHC and immune response genes (Fig. 6, arrows 3 and 4). (A) Dynamics of WT gene expression. (B) Dynamics of IFNAR^{-/-} gene expression. Nodes and edges are colored according to the legend in Fig. 6.

metabolism were strongly upregulated in IFNAR^{-/-} DC as well as a group of UDP-glucouronosyltransferases, which are key antioxidants and detoxifiers of the cell (Fig. 6B, arrow 6). Based on these data, we postulated that the DC lacking IFNAR might be differentially susceptible to oxidative damage. To determine if IFNAR^{-/-} DCs were more or less susceptible to oxidative stress, WT and IFNAR^{-/-} DCs were incubated with 100 μ M H₂O₂ in the presence or absence of Poly I:C and cell death was measured by Annexin V/7AAD staining. While H₂O₂ caused cell death in both populations, no significant difference was observed between WT and IFNAR^{-/-} DC (data not shown). Thus, we have no evidence to support this hypothesis to date.

Finally, WT and IFNAR^{-/-} cells shared an active subnetwork associated with motility, focal adhesions, and interactions with the extracellular matrix (Fig. 6A, B, arrow 7). Some nodes were common between WT and IFNAR^{-/-}, and some were distinct. Both WT and IFNAR^{-/-} DCs had active nodes in several Syndecan genes, including Sdc1 and Sdc4; however, the patterns of expression were quite different. In WT, Sdc1 and Sdc4 were generally upregulated across the time course in concert with another node, Sdc3, which was not active in IFNAR^{-/-} DC. In IFNAR^{-/-} DC, Sdc1 was upregulated, while Sdc4 was downregulated late in the time course. Syndecan 1 and 4 have been reported to mediate maturation-associated changes in DC motility (Averbeck and others 2007). Thus, these findings imply differences in WT and IFNAR^{-/-} DC motility upon maturation. Most strikingly, however, there was a large group of

collagen genes (Col1a1, Col1a2, Col3a1, Col4a1, Col5a2, Col6a1, and Col6a2) that were upregulated in the IFNAR^{-/-} DCs that were completely lacking in the WT DCs. This novel finding suggests that the type I interferon response impacts the expression and/or deposition of collagen matrix upon maturation. This is important in light of several studies examining the differential behavior of DC in or outside of a collagen matrix (Gunzer and others 2000; Methe and others 2007). While this remains a controversial issue, collagen production and cellular interactions with the extracellular matrix are clearly key to the migration of DC and are likely to be critical *in vivo*. These are active areas of investigation in our laboratory.

Discussion

The functions associated with the DC maturation response in WT DC are summarized in Fig. 8. The upregulated functions included MHC, costimulation, antigen process, proteolysis, lysosomal transport, cytokine signaling, and apoptosis (Fig. 8, left panel). These functions represent many of what we know to be hallmarks of the DC maturation response, optimizing the T-cell stimulation potential. In WT cells, the downregulated response involved many more genes, yet the functions associated with this response were generally associated with RNA—processing, transport, catabolism, and ribosome biogenesis (Fig. 8, left panel). Thus, DC shut down gene expression and translation at the later stages of Poly I:C-induced maturation.

FIG. 8. Functions associated with Poly-IC induced gene expression and regulation by IFNAR. Based on the collective results of the analysis of this data set, the most prominent upregulated functions in the WT DC were involved in T-cell stimulation—MHC, costimulatory molecules, antigen processing, and cytokine signaling and regulation (*left panel*). Other prominent upregulated functions in WT DC were lysosomal transport and apoptosis. The functions associated with the large downregulated response in the WT DC were predominantly involved in gene expression—RNA processing, transport and metabolism, as well as ribosome biogenesis (*lower left panel*). IFNAR^{-/-} DC showed a very distinct upregulated response to poly I:C stimulation dominated by oxidative stress response and glutathione synthesis functions, also prominent in IFNAR^{-/-} DC (*right panel*). Metabolism of carbohydrate and lipids was also prominent in the upregulated response of IFNAR^{-/-} cells as well as collagen deposition, apoptosis, and DNA repair. The downregulated response in IFNAR^{-/-} DC was dominated by cell division-associated functions, including DNA replication, mitosis, and division.

The response induced in IFNAR^{-/-} DC by Poly I:C was quite different than WT cells (Fig. 8, right panel). The upregulated response in these cells was lacking the strong antigen processing and presentation functions of WT and instead demonstrated a prominent protective response against oxidative stress, including glutathione synthesis. Other upregulated functions observed in IFNAR^{-/-} DC included carbohydrate and lipid metabolism and catabolism. We also observed a significant set of functions associated with collagen deposition and interaction with the extracellular matrix, apoptosis, and DNA repair in IFNAR^{-/-} DCs that were not observed in WT (Fig. 8, right panel). Thus, these upregulated responses observed only in IFNAR^{-/-} cells appear to be negatively regulated by IFNAR. The downregulated response in IFNAR^{-/-} DCs was very small compared to WT cells and was associated with functions, including DNA replication, mitosis, and cell division. Therefore, in the absence of IFNAR, the DC shut down cell division rather than the gene expression shutdown observed in WT.

Role of IFNAR in the downregulated gene expression program induced by Poly I:C

We have previously reported that many of the genes involved in the downregulated pattern have a role in biogenesis

and protection from cell death. These processes appear to be shut down in the late stages of maturation, suggesting terminal differentiation and initiation of apoptosis. Because this response is lacking or less prominent in the absence of IFNAR, we postulate that IFNAR-mediated signals likely regulate the shutdown of biogenesis and/or induction of apoptosis. One of the most prominent aspects of the gene expression program induced by Poly I:C in WT DC is the large number of genes whose expression is downregulated (Olex and others 2010). Downregulation of the transcriptomic fingerprint of quiescent DC has also been observed *in vivo* upon viral infection and is a phenomenon common to several DC subsets (Manh and others 2013). However, in DC lacking IFNAR, this response is largely missing and, instead, the IFNAR^{-/-} DC program consists primarily of upregulation. The major function annotations associated with the downregulated response in WT were RNA related, including processing, binding, transport, catabolism, and so on (Tables 2 and 3). Thus, RNA metabolism is downregulated during Poly I:C induced maturation, a process that is dependent on type I interferon signaling. One might hypothesize that lowering the SLR filtering level may identify many of the downregulated genes that are lacking in the IFNAR response. However, the reliability of microarray data at lower expression values is not high. We could not reliably detect a downregulated response of lower magnitude in these microarray data.

In contrast to the great difference in numbers of genes in the downregulated response observed between WT and IFNAR^{-/-} DC, the upregulated response was almost identical in gene number (611 in WT vs. 609 in IFNAR^{-/-}) in both cell types. However, the identities of these upregulated genes were quite distinct (only 79 genes overlapped, Fig. 4). The notable differences between the program of upregulated genes in WT versus IFNAR^{-/-} are that WT had a much more significant group of genes involved in intracellular transport, proteolysis, and ubiquitination, while DC lacking IFNAR had a more prominent group of annotations associated with metabolism and oxidative stress.

MHC, antigen processing and presentation, and costimulatory function

Our study first showed the profound impact of IFNAR on the maturation and function of DCs *in vitro*. DCs lacking IFNAR were compromised in their expression of costimulatory molecules, cytokines, MHC, and in their ability to stimulate T-cell activation (Figs. 1–3). These cellular readouts were further supported by gene expression data showing deficiency in the expression of a variety of MHC-associated genes in IFNAR^{-/-} DC. These findings are in agreement with a previous study in lupus-prone NZM mice showing that DC number and function are decreased in mice lacking IFNAR (Agrawal and others 2009). While the role for IFNAR in expression of MHC class I is well established (Langer and others 1992; Cheng and others 2004; Pogue and others 2004; Moro and others 2011), its role in antigen processing and cross-presentation is an area of active investigation (Lopez-Albaitero and others 2009; Lattanzi and others 2011; Simmons and others 2012; Quinn and others 2013). Consistent with our subnetwork analysis, which showed an IFNAR-dependent upregulation of MHC class I and its associated peptide transporters and chaperones, several reports have shown the enhancing activity of type I

interferon on antigen processing and presentation in the MHC class I pathway as well as the cross-presentation pathway (Lopez-Albaitero and others 2009; Lattanzi and others 2011).

The impact of type I interferon exposure in DC development and function has now been well established (reviewed in (Gessani and others 2014)). The type I interferon response is also known to impact antigen processing and presentation by regulating MHC expression (Simmons and others 2012). Our data extend these findings by suggesting a role for type I interferon signaling in the lysosomal transport and proteolytic activity required for antigen processing and presenting function of these cells.

The cytokine response of DC maturation regulated by IFNAR

Our cytokine data are also consistent with previous studies showing modest production of IFN- β in the absence of the amplification that comes with IFNAR signaling, (Gautier and others 2005) and that IL-12 is not strongly induced by Poly I:C treatment without additional IFN- γ treatment (Gautier and others 2005). While these findings are consistent with previous studies, this targeted analysis of secreted cytokines could not have predicted how many other cytokine genes' and cytokine receptor genes' expressions were regulated by IFNAR. Perhaps the most different response in the WT versus IFNAR^{-/-} subnetworks identified was a large group of cytokine receptors (Fig. 6). Members of this large network were generally upregulated upon stimulation of WT cells, but no detectable response was observed in these genes in IFNAR^{-/-} DC.

The prominence of this well-known network, including many nodes that are hallmarks of the response to poly I:C in WT cells (Scumpia and others 2005; Flur and others 2009; Hamasaki and others 2010; Durafour and others 2011), validates the subnetwork analysis and suggests its utility in identifying novel networks associated with this process. The striking absence of this response in the IFNAR^{-/-} DC highlights the role IFNAR signaling plays in the expression of a large group of cytokine receptors upon DC maturation. Thus, in the absence of IFNAR signaling, many cells would remain insensitive to these proinflammatory cytokine signals. While the cytokine response was truncated in IFNAR^{-/-} DC, we did observe a large subnetwork of NF- κ B transcription factors in these cells at early time points following stimulation. These data are consistent with previous reports as well as our own observations that IFNAR^{-/-} DCs are competent at NF- κ B-dependent functions such as inflammatory cytokine production following Poly I:C treatment (IL-6 production, Fig. 1) (Jia and others 2009; Pantel and others 2014). Thus, the impact of IFNAR on a broad array of the cytokine response was much greater than expected. Collectively, these findings support the notion that the type I interferon receptor significantly impacts the DC maturation response induced by Poly I:C by regulating costimulatory activity, MHC expression, and cytokine production. Our findings further support a major role for the type I IFN receptor (IFNAR) in several key DC functions that determine their ability to interact with and activate T cells, including antigen presentation, costimulation, and cytokine production.

Novel subnetworks of gene expression and regulation by IFNAR

Among the novel subnetworks revealed by our network analysis, Nucleotide metabolism/repair was one of the most unexpected. This particular group of genes has not previously been reported to be associated with DC maturation. However, there have been recent reports regarding the role of other DNA repair proteins and their relationship to DC maturation (Aldinucci and others 2007; Briegert and others 2007; Bauer and others 2011). It has been recently reported that monocytes and DCs become more susceptible to oxidative stress upon maturation due, in part, to decreased DNA repair pathways (Bauer and others 2011). Based on the patterns of transcriptional up- and downregulation observed within this group, it is hard to make predictions about exactly how the DNA repair machinery would behave in WT DCs during maturation. These data open up interesting avenues for further investigation.

One of the subnetworks uniquely observed in the IFNAR^{-/-} DC was involved in redox reactions.

The role of glutathione synthesis and metabolism has been reported for DC maturation in WT cells (Mizuashi and others 2005; Yamada and others 2006; Kim and others 2007; Khatri and others 2009; D'Angelo and others 2010). More recently, an elegant study by Pantel and others (2014) demonstrated that Poly I:C induces a metabolic switch in the WT DC from oxidative phosphorylation to glycolysis with a corresponding change in transcriptional profile. Intriguingly, their study also showed that this metabolic switch was dependent on IFNAR.

Taken together, these studies have revealed a number of novel and important insights regarding the gene expression program underlying the DC maturation process. First, it is clear that IFNAR mediates a large portion of the response induced by poly I:C. In fact, in DCs lacking IFNAR, the number of genes whose expression was significantly modulated (positively or negatively) was less than half of that observed in WT. Strikingly, this receptor was required for the large downregulated gene expression program. Second, our computational modeling analysis yielded several subnetworks that have known to be central in the DC maturation program. This not only validated our approach but also provided a more detailed understanding of the genes within these subnetworks and their regulation by the type I interferon response. Finally and perhaps most importantly, integration of static networks with the temporal gene expression data collected here has revealed several novel subnetworks of gene expression, some of which are sensitive to the type I interferon response. Determining the roles of the members of these novel subnetworks such as nucleotide metabolism and oxidative stress will not only provide a more comprehensive understanding of this important process but also potential targets for clinical therapies.

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Address correspondence to:

Dr. Elizabeth M. Hiltbold
Department of Biological Sciences
Auburn University
101 Life Sciences Building
Auburn, AL 36849

E-mail: ehs0009@auburn.edu

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