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This information is current as of February 26, 2022.

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*J Immunol* 2012; 188:902-915; Prepublished online 5 December 2011;

doi: 10.4049/jimmunol.1102797

<http://www.jimmunol.org/content/188/2/902>

**Supplementary Material** <http://www.jimmunol.org/content/suppl/2011/12/05/jimmunol.1102797.DC1>

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# Plasmacytoid Dendritic Cells and C1q Differentially Regulate Inflammatory Gene Induction by Lupus Immune Complexes

Deanna M. Santer,\* Alice E. Wiedeman,\* Thomas H. Teal,<sup>†</sup> Pradipta Ghosh,<sup>†</sup> and Keith B. Elkon\*,<sup>†</sup>

Immune complexes (ICs) play a pivotal role in causing inflammation in systemic lupus erythematosus (SLE). Yet, it remains unclear what the dominant blood cell type(s) and inflammation-related gene programs stimulated by lupus ICs are. To address these questions, we exposed normal human PBMCs or CD14<sup>+</sup> isolated monocytes to SLE ICs in the presence or absence of C1q and performed microarray analysis and other tests for cell activation. By microarray analysis, we identified genes and pathways regulated by SLE ICs that are both type I IFN dependent and independent. We also found that C1q-containing ICs markedly reduced expression of the majority of IFN-response genes and also influenced the expression of multiple other genes induced by SLE ICs. Surprisingly, IC activation of isolated CD14<sup>+</sup> monocytes did not upregulate CD40 and CD86 and only modestly stimulated inflammatory gene expression. However, when monocyte subsets were purified and analyzed separately, the low-abundance CD14<sup>dim</sup> ("patrolling") subpopulation was more responsive to ICs. These observations demonstrate the importance of plasmacytoid dendritic cells, CD14<sup>dim</sup> monocytes, and C1q as key regulators of inflammatory properties of ICs and identify many pathways through which they act. *The Journal of Immunology*, 2012, 188: 902–915.

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the presence of high titer autoantibodies directed against self nucleoproteins (reviewed in Ref. 1). Some of these Abs interact with Ags to form immune complexes (ICs) that deposit in the kidneys, skin, and vasculature (2, 3). ICs also directly engage FcγRs expressed on macrophages and neutrophils, resulting in the release of proinflammatory cytokines, proteolytic enzymes, and reactive oxygen intermediates (4). Thus, ICs, especially after complement activation, are thought to be the predominant inducers of tissue injury in SLE (3, 5).

Despite these findings, individuals with loss-of-function mutations of the first complement component, C1q, almost invariably develop SLE (reviewed in Ref. 6). This "lupus paradox" (7), in which complement activation promotes tissue injury, yet complement deficiency predisposes to SLE, has, in part, been reconciled by studies demonstrating the protective role of classical complement pathway components (C1–4) in facilitating the clearance of lupus Ags (apoptotic debris) (8–10). In contrast, it is the com-

plement components downstream of C3, especially release of C3a and C5a that promote chemotaxis and inflammation as well as deposition of C5b-9 together, that promote tissue injury (11–13).

More recently, C1q has been shown to protect against SLE by a separate effect, via preventing the stimulation of type I IFN (14, 15). We observed that, in the absence of C1q, ICs bind to FcγRII on plasmacytoid dendritic cells (pDCs) and potently stimulate IFN-α production, whereas C1q-containing ICs (C1q-ICs) bound predominantly to monocytes, and IFN-α production by pDCs was markedly attenuated (15). Although C1q-ICs did not stimulate monocytes to produce soluble factors such as IL-10 or TNF-α that could explain IFN-α suppression, we could not exclude the possibility that ICs induce a ligand on monocytes that engages one of the many inhibitory receptors on pDCs.

To obtain comprehensive data of genes differentially regulated by exposure of blood cells to SLE ICs, to determine how this profile is altered in the presence of C1q-ICs, and to evaluate the inflammatory properties of ICs on monocytes in the absence of pDCs, we performed gene expression analysis of peripheral blood as well as isolated monocytes stimulated by ICs or C1q-ICs. Our results enable comparison between the in vitro effects of ICs and ex vivo inflammatory gene transcript profiles as well as those genes regulated by C1q. They also show limited CD14<sup>+</sup> monocyte stimulation by ICs in the absence of pDCs and suggest relevant genes and pathways that should prove productive for future investigation of SLE pathogenesis.

## Materials and Methods

### Reagents

Purified C1q protein was purchased from Complement Technology. Neutralizing Ab to IFN-α was purchased from Millipore. Loxoribine was purchased from Invivogen. All reagents had <0.06 EU/ml endotoxin by *Limulus* amebocyte lysate clot assay (Cape Cod Associates).

### Patients

All SLE patients fulfilled the American College of Rheumatology 1982 revised criteria for the classification of SLE (16). All serum samples were collected with the respective institutions' review board approval.

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Received for publication September 27, 2011. Accepted for publication November 3, 2011.

This work was supported by National Institutes of Health Grants AR48796 and NS065933. D.M.S. was supported by a Natural Sciences and Engineering Research Council of Canada postgraduate scholarship and a Kirkland scholarship.

The microarray data presented in this article have been submitted to the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32285>) under Series accession number GSE32285.

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The online version of this article contains supplemental material.

Abbreviations used in this article: C1q-IC, C1q-containing immune complex; GOI, gene of interest; IC, immune complex; IP-10, IFN-γ-inducible protein-10; ISG, IFN-stimulated gene; MFI, mean fluorescent intensity; pDC, plasmacytoid dendritic cell; qRT-PCR, quantitative real-time PCR; SLC16A10, solute carrier family 16, member 10; SLE, systemic lupus erythematosus.

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### Cell purification

PBMCs were prepared from healthy human donors or SLE patients using Ficoll-Paque density gradient centrifugation. For normal donor experiments, a different healthy donor was used for each independent experiment. In certain experiments, pDCs were depleted from PBMCs using BDCA-4 magnetic beads (Miltenyi Biotec) with <0.03% remaining in each experiment. As an additional control, PBMCs were mock depleted by incubating cells without beads, but still placed through the magnetic column. Total monocytes were purified from PBMCs by positive selection with CD14 magnetic beads (Miltenyi Biotec) with consistent purities of >95% and undetectable percentages of contaminating pDCs. In certain experiments, monocyte subsets were sorted to purities of >90–95% using methods described by others (17). Briefly, cells were stained with the following fluorescently labeled Abs (all from BioLegend, unless otherwise noted): CD19-PE (clone HIB19), CD56-PE (clone MEM-188), NKp46-PE (clone 9E2), CD15-PE (clone H198), CD2-PE (clone RPA-2.10), HLA-DR-PerCp/Cy5.5 (clone L243), CD16-Alexa Fluor 488 (clone 3G8), and CD14-allophycocyanin-Alexa Fluor 780 (clone 61D3; eBioscience). Cells were gated for the monocyte population that lacked the PE stain (B cells, NK cells, granulocytes, and T cells), but which was HLA-DR<sup>+</sup>; this was further divided into three monocyte subsets that included the CD14<sup>+</sup>CD16<sup>−</sup>, CD14<sup>+</sup>CD16<sup>dim</sup>, and CD14<sup>+</sup>CD16<sup>+</sup> subsets that were sorted and collected live using a FACSria flow cytometer (BD Biosciences).

### Cell stimulation

To form ICs, high dilutions of SLE serum or purified SLE IgG (5–15 µg/ml) were used as a source of autoantibodies, and freeze-thawed U937 cells were used as autoantigen, as described previously (15, 18, 19). Briefly, SLE serum (diluted 1:1000–1:2000 with RPMI 1640 media) was mixed with U937 freeze-thawed cell extract. Cell debris was removed by centrifugation, and the extract was added to the cell type being tested at a 1% v/v concentration. As reported previously, IFN-α production was RNA, FcγRIIIa, and TLR7 dependent (19). Although many SLE patient sera were used in the course of this study, the two sera used to make ICs for the microarray experiments both had the following autoantibody profile: Sm/RNP<sup>+</sup>, Ro<sup>−</sup>, La<sup>−</sup>, dsDNA<sup>+</sup>. ICs were added to normal PBMCs (5 × 10<sup>5</sup>/well) and left unprimed or primed with type I IFN and GM-CSF, as previously described (15, 18, 19). In our culture system, IFN-α is only produced by pDCs as Abs to BDCA-2 abrogated IFN-α production, as described previously (20), and IFN-α was not detectable in pDC-depleted PBMCs or purified monocyte cultures (data not shown). U937 cells were determined to be free of mycoplasma contamination using e-Myco Mycoplasma PCR detection kit (iNtRON Biotechnology). Toxicity of added inhibitors was monitored by flow cytometry with LIVE/DEAD I/R (Invitrogen).

### Microarray

Unprimed total PBMCs from two different healthy donors were plated in 24-well plates at 2 × 10<sup>6</sup>/well in one well and left unstimulated or stimulated with SLE ICs formed, as above, with or without C1q for ~5 h. For monocyte stimulations, a total of 1 × 10<sup>6</sup> monocytes per condition from two different healthy donors was plated in two wells of a 96-well plate and left unstimulated or stimulated, as above. Cells were washed three times in PBS, and total RNA was isolated using a Qiagen RNeasy kit with on column DNase treatment (Qiagen). RNA (400 ng) was checked for high quality using an Agilent 2100 Bioanalyzer (Agilent Technologies) by the Fred Hutchinson Cancer Research Center's Genomics Resource, and samples were labeled and hybridized to Illumina's Human Ref-8v3 Expression BeadChips following the manufacturer's recommendations (Illumina). BeadChips were scanned on an Illumina Bead Array Reader System.

### Microarray data analysis

Probe-level results were generated in GenomeStudio Data Analysis Software's Gene Expression Module (GSGX) Version 1.5.4 (Illumina). BeadChip results were background corrected and quantile normalized using the Bioconductor package lumi (21). A small offset was applied across all arrays to bring values above 0. Data were discarded from further analysis if the processed signal across pairwise comparisons was below 3 times the mean intensity values of the negative control probes on the BeadChips. Microarray data have been deposited in the Gene Expression Omnibus database and are accessible through the Gene Expression Omnibus Series accession number GSE32285 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32285>). Genes listed in tables had gene expression changes upregulated or downregulated >1.5-fold compared with unstimulated controls in both donors, which allowed detailed pathway analysis by In-

genuity software. In some cases, changes in gene expression of 2-fold or greater were analyzed separately. Functional analysis of differentially expressed genes was performed using Ingenuity pathways analysis (Ingenuity Systems). Some genes considered were not eligible for Ingenuity pathway analysis, but are included in the Venn diagrams. The significance of the association between the data set and the canonical pathway was measured using Fisher's exact test to calculate a *p* value representing the probability that the association between the transcripts in the data set and the canonical pathway was explained by chance alone. Canonical functions were also determined, and networks were overlaid with expression data from the data set. IFN-stimulated genes (ISGs) were identified by examining eight publications (22–29) plus the interferome.org database specifically for type I IFN (30).

### Quantitative real-time PCR

First-strand cDNA was generated with the RNA-to-cDNA kit (Applied Biosystems) using random primers. cDNA was diluted to an equivalent of 0.2 ng/µl total RNA, and 8 µl was used per reaction. Primers for the reference gene (18S) and genes of interest (GOI) were synthesized by Integrated DNA Technologies. After melt curve and standard curve analysis, primers were diluted to the most efficient concentrations for quantitative real-time PCR (qRT-PCR) using molecular grade water. BLAST results of the primers show specific sequence homology only to the reference gene or GOI. Reactions in duplicate or triplicate (20 µl) were run on an ABI Fast 7500 system using a 1:1 mix of template/primer to SensiMix SYBR low-ROX master mix (Bioline). Relative quantification was calculated using the 2<sup>−ddCT</sup> method with unstimulated cells as baseline to determine fold changes for each GOI. Primer sequences for genes are shown in Supplemental Table I.

### Cytokine detection

IFN-α levels in supernatants were quantified by ELISA using commercially available Abs, as described (15). IFN-γ-inducible protein-10 (IP-10), MCP-1, MCP-3, IL-1RA, IL-8, IL-10, IL-12p40, IL-1β, and MIP-1α levels were quantified in supernatants using a custom 9-plex Bio-Plex kit from Bio-Rad. TNF-α was measured in supernatants by ELISAs with Ab pairs from BioLegend.

### Flow cytometry for activation markers

Cells were left untreated (medium) or ICs were added to total PBMCs, pDC-depleted PBMCs, or purified monocytes as part of the stimulation assay described above. At least two different SLE patient ICs were added for each independent experiment. After 2 d of stimulation, cells were washed and surface stained to identify monocytes (CD14 clone HCD14; BioLegend) and the level of expression of the activation markers CD86 (clone IT2.2; BioLegend) and CD40 (clone 5C3; BioLegend). Monocyte subsets were gated for activation analysis, as described above. The mean fluorescent intensities (MFI) for each marker are plotted.

### Statistical analysis

Statistical significance between groups was determined by Mann–Whitney *U* test, unpaired/paired *t* test, or one-way ANOVA, where appropriate. Correlations between parameters were assessed using the Pearson correlation analysis and linear regression analysis. A *p* value <0.05 was considered significant. Graphs and statistical analyses were performed using Prism software (v 4; GraphPad Software).

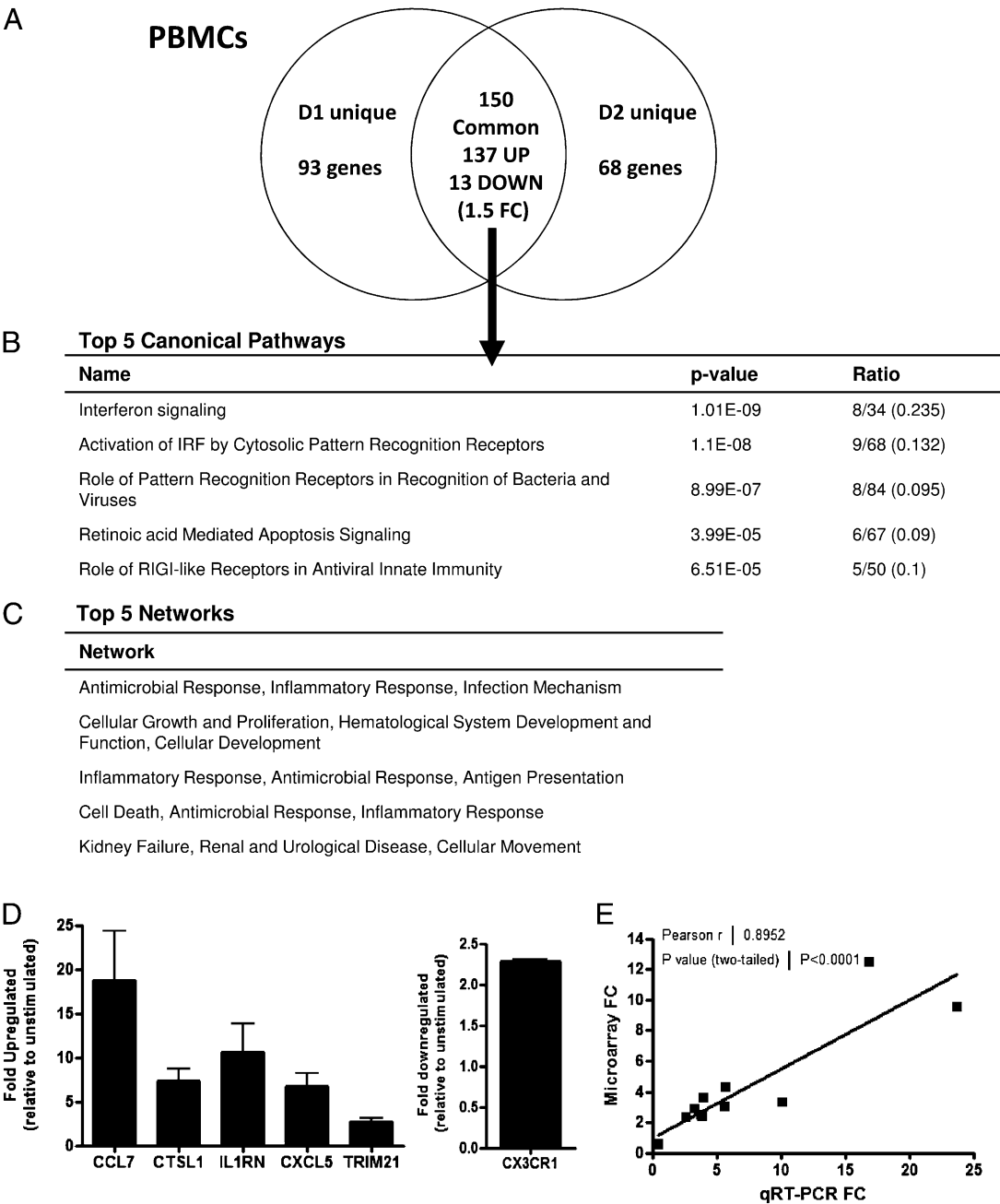
## Results

### RNA-containing SLE ICs predominantly induce ISG expression in PBMCs

ICs have been implicated in the induction of tissue inflammation in SLE and many other diseases (reviewed in Ref. 31). Although it is now well documented that SLE ICs stimulate IFN-α production by pDCs (15, 32–34), the full range of cytokine and other inflammatory markers produced by whole blood mononuclear cells has not been studied by unbiased comprehensive approaches such as microarray. To identify common genes stimulated by SLE ICs, we incubated total PBMCs from two different donors (responder cells), each with a different SLE IC, and then examined gene expression by microarray analysis after 6 h. Using Ingenuity software to analyze genes for pathway analysis, 243 genes and 218 annotated genes were upregulated or downregulated >1.5-fold by SLE

ICs in donors D1 and D2, respectively. Of the 150 genes that were common between the two donors, 137 genes were upregulated and 13 genes downregulated >1.5-fold (Fig. 1A, Table I, Supplemental Table II). We observed a striking induction of ISGs such that 43 of 50 top genes regulated have previously been linked to type I IFNs (bold lettering, Table I) (see *Materials and Methods* for publications used to determine ISGs). Although two different donors and ICs were used, the top genes induced were very similar in the responder cells with identical top five canonical pathways and network functions (Fig. 1B, 1C). We validated six genes

regulated >1.5-fold by qRT-PCR using an additional two donors for a total of four independent experiments and found a significant correlation between the fold induction obtained from qRT-PCR and the microarray data with those six genes tested (Fig. 1D, 1E). Although 70% of all genes regulated were type I IFN ISGs, we also identified numerous other genes regulated by SLE ICs that include cytokines (e.g., IL-1 family member 7 [IL1F7], increased 5-fold), transcription factors (e.g., EGR1, reduced almost 3-fold), and cell surface receptors (e.g., CLEC12A, reduced ~2-fold) (Table I).



**FIGURE 1.** Differential expression of genes and pathways in PBMCs after exposure to SLE ICs. *A–C*, SLE ICs were incubated with unprimed normal donor PBMCs for 5 h. Following RNA isolation, gene expression was quantified by microarray, as described in *Materials and Methods*. *A*, Summary of genes regulated >1.5-fold compared with unstimulated controls with the intersection representing overlapping genes in two different donors (D1 and D2). *B* and *C*, The top five canonical pathways (*B*) and networks (*C*) are shown following PBMC IC stimulation. *D*, Five upregulated and one downregulated gene from the microarray data were quantified by qRT-PCR with four independent normal PBMC donor stimulations. *E*, The fold changes from the microarray and qRT-PCR for the same samples were plotted, and the Pearson correlation coefficient was calculated ( $r = 0.8952$ ,  $p < 0.0001$ ). FC, fold change relative to unstimulated controls.



Table I. Top 50 genes changed upon addition of SLE ICs to total PBMC cultures

Gene	Gene Name	PROBE_ID <sup>a</sup>	REFSEQ_ID	D1 FC <sup>b</sup>	D2 FC <sup>b</sup>	Mean FC
<b>RSAD2<sup>c</sup></b>	<b>Radical S-adenosyl methionine domain containing 2</b>	ILMN_1657871	NM_080657.4	40.253	10.486	25.370
<b>CXCL10</b>	<b>Chemokine (C-X-C motif) ligand 10</b>	ILMN_1791759	NM_001565.2	25.672	14.578	20.125
<b>IFIT1</b>	<b>IFN-induced protein with tetratricopeptide repeats 1</b>	ILMN_1707695	NM_001548.3	29.491	8.374	18.932
<b>IFIT3</b>	<b>IFN-induced protein with tetratricopeptide repeats 3</b>	ILMN_1701789	NM_001031683.1	27.491	8.199	17.845
<b>CCL8</b>	<b>Chemokine (C-C motif) ligand 8</b>	ILMN_1772964	NM_005623.2	23.576	4.798	14.187
<b>ISG15</b>	<b>ISG15 ubiquitin-like modifier</b>	ILMN_2054019	NM_005101.1	16.817	6.977	11.897
<b>IFIT2</b>	<b>IFN-induced protein with tetratricopeptide repeats 2</b>	ILMN_1739428	NM_001547.4	15.324	7.076	11.200
<b>IFI44L</b>	<b>IFN-induced protein 44-like</b>	ILMN_1723912	NM_006820.1	13.079	4.058	8.568
<b>IL1RN</b>	<b>IL-1 receptor antagonist</b>	ILMN_1774874	NM_173843.1	12.476	4.305	8.390
<b>DEFB1</b>	<b>Defensin, <math>\beta</math>1</b>	ILMN_1686573	NM_005218.3	10.839	5.185	8.012
<b>OAS1</b>	<b>2',5'-oligoadenylate synthetase 1, 40/46 kDa</b>	ILMN_1658247	NM_002534.2	9.650	4.972	7.311
<b>OASL</b>	<b>2',5'-oligoadenylate synthetase-like</b>	ILMN_1681721	NM_003733.2	9.018	4.406	6.712
<b>HES4</b>	<b>Hairy and enhancer of split 4 (<i>Drosophila</i>)</b>	ILMN_1653466	NM_021170.2	9.072	4.227	6.650
<b>HERC5</b>	<b>Hect domain and RLD 5</b>	ILMN_1729749	NM_016323.2	8.519	4.132	6.326
<b>IFI44</b>	<b>IFN-induced protein 44</b>	ILMN_1760062	NM_006417.3	8.264	3.868	6.066
<b>CCL7</b>	<b>Chemokine (C-C motif) ligand 7</b>	ILMN_1683456	NM_006273.2	9.552	2.506	6.029
<b>TNFSF10</b>	<b>TNF (ligand) superfamily, member 10 (TRAIL)</b>	ILMN_1801307	NM_003810.2	8.288	3.757	6.023
<b>IFI6</b>	<b>IFN, <math>\alpha</math>-inducible protein 6</b>	ILMN_2347798	NM_022872.2	7.613	3.613	5.613
<b>IFITM3</b>	<b>IFN-induced transmembrane protein 3 (1-8U)</b>	ILMN_1805750	NM_021034.2	6.337	4.233	5.285
<b>IL1F7</b>	<b>IL-1 family, member 7 (<math>\zeta</math>)</b>	ILMN_1697710	NM_014439.3	7.599	2.782	5.191
<b>EPSTI1</b>	<b>Epithelial stromal interaction 1 (breast)</b>	ILMN_2388547	NM_033255.2	6.860	3.061	4.960
<b>MX2</b>	<b>Myxovirus (influenza virus) resistance 2</b>	ILMN_2231928	NM_002463.1	6.736	3.114	4.925
<b>MX1</b>	<b>Myxovirus (influenza virus) resistance 1</b>	ILMN_1662358	NM_002462.2	6.253	3.077	4.665
<b>DDX58</b>	<b>DEAD (Asp-Glu-Ala-Asp) box polypeptide 58</b>	ILMN_1797001	NM_014314.3	5.045	3.937	4.491
<b>STAT1</b>	<b>Signal transducer and activator of transcription 1, 91 kDa</b>	ILMN_1690105	NM_007315.2	5.920	3.058	4.489
<b>HERC6</b>	<b>Hect domain and RLD 6</b>	ILMN_1654639	NM_017912.3	5.535	3.294	4.414
<b>PRIC285</b>	<b>Peroxisomal proliferator-activated receptor A complex 285</b>	ILMN_1787509	NM_033405.2	5.503	2.995	4.249
<b>RTP4</b>	<b>Receptor (chemosensory) transporter protein 4</b>	ILMN_2173975	NM_022147.2	4.723	3.673	4.198
<b>EIF2AK2</b>	<b>Eukaryotic translation initiation factor 2-<math>\alpha</math> kinase 2 (PKR)</b>	ILMN_1706502	NM_002759.1	5.372	2.719	4.046
<b>SAMD9L</b>	<b>Sterile <math>\alpha</math> motif domain containing 9-like</b>	ILMN_1799467	NM_152703.2	4.469	3.341	3.905
<b>TMEM158</b>	<b>Transmembrane protein 158 (gene/pseudogene)</b>	ILMN_1792455	NM_015444.2	2.250	5.369	3.810
<b>CLDN23</b>	<b>Claudin 23</b>	ILMN_1725338	NM_194284.2	3.611	3.956	3.783
<b>OAS2</b>	<b>2'-5'-oligoadenylate synthetase 2, 69/71 kDa</b>	ILMN_1736729	NM_002535.2	4.777	2.711	3.744
<b>XAF1</b>	<b>XIAP-associated factor 1</b>	ILMN_2370573	NM_199139.1	4.859	2.608	3.734
<b>LY6E</b>	<b>Lymphocyte Ag 6 complex, locus E</b>	ILMN_1695404	NM_002346.1	4.375	3.063	3.719
<b>DHRS9</b>	<b>Dehydrogenase/reductase (SDR family) member 9</b>	ILMN_2384181	NM_005771.3	3.033	4.273	3.653
<b>CTSL1</b>	<b>Cathepsin L1</b>	ILMN_1812995	NM_001912.3	3.583	3.634	3.609
<b>IFIT5</b>	<b>IFN-induced protein with tetratricopeptide repeats 5</b>	ILMN_1696654	NM_012420.1	4.456	2.587	3.522
<b>GBP1</b>	<b>Guanylate-binding protein 1, IFN inducible, 67 kDa</b>	ILMN_2148785	NM_002053.1	4.504	2.497	3.501
<b>IFIH1</b>	<b>IFN induced with helicase C domain 1</b>	ILMN_1781373	NM_022168.2	4.411	2.455	3.433
<b>IFI35</b>	<b>IFN-induced protein 35</b>	ILMN_1745374	NM_005533.2	3.963	2.777	3.370
<b>IRF7</b>	<b>IFN regulatory factor 7</b>	ILMN_2349061	NM_004029.2	3.846	2.792	3.319
<b>LAP3</b>	<b>Leucine aminopeptidase 3</b>	ILMN_1683792	NM_015907.2	4.004	2.458	3.231
<b>EMP1</b>	<b>Epithelial membrane protein 1</b>	ILMN_1801616	NM_001423.1	3.087	3.215	3.151
<b>DDX60</b>	<b>DEAD (Asp-Glu-Ala-Asp) box polypeptide 60</b>	ILMN_1795181	NM_017631.4	3.582	2.443	3.012
<b>CXCL5</b>	<b>Chemokine (C-X-C motif) ligand 5</b>	ILMN_2171384	NM_002994.3	3.042	2.894	2.968
<b>NT5C3</b>	<b>5'-nucleotidase, cytosolic III</b>	ILMN_2352121	NM_001002010.1	3.400	2.454	2.927
<b>JUP</b>	<b>Junction plakoglobin</b>	ILMN_1733811	NM_002230.1	3.149	2.676	2.912
<b>EGR1</b>	<b>Early growth response 1</b>	ILMN_1762899	NM_001964.2	-2.878	-2.902	-2.890
<b>MT2A</b>	<b>Metallothionein 2A</b>	ILMN_1686664	NM_005953.2	3.755	2.018	2.886

<sup>a</sup>Probe ID from Illumina Human Ref-8v3 Expression BeadChips.<sup>b</sup>FC, fold change relative to unstimulated controls. D1 and D2, donor 1 and donor 2. Positive numbers indicate fold upregulated; negative numbers indicate fold downregulated.<sup>c</sup>Bold lettering indicates type I ISGs.

### The addition of C1q to ICs reduced the expression of ISGs and modulated the expression of multiple other genes

We recently observed that the addition of C1q to SLE ICs markedly reduced IFN- $\alpha$  stimulation and that this effect was explained, in part, by monocytes “stealing” the ICs away from pDCs (15). To examine the effect of C1q on ISGs and also determine what other pathways were altered by the presence of C1q bound to ICs (C1q-ICs), we repeated the microarray analysis as above, except that we compared SLE IC stimulation in the presence or absence of C1q. In

total, 241 genes were upregulated or downregulated >1.5-fold by C1q-ICs compared with IC stimulation alone with 67 genes upregulated and 174 genes downregulated (Table II, Supplemental Table III). The top 50 genes regulated 1.5-fold or greater are shown in Table II. Upon analysis of the top canonical pathways regulated by C1q, the top three were directly related to type I IFNs (Fig. 2A), and the top networks included many of these genes as well (Fig. 2B). A strong correlation between the microarray and qRT-PCR results was observed with three genes and four different PBMC

Table II. Top 50 genes changed upon addition of C1q to SLE ICs in total PBMC cultures

Gene	Gene Name	REFSEQ_ID	Mean IC FC <sup>a</sup>	Mean C1q-IC FC <sup>a</sup>	C1q-IC/IC Ratio <sup>b</sup>	FC C1q-IC/IC <sup>c</sup>
RNASE1	RNase, RNase A family, 1 (pancreatic)	NM_198235.1	1.820	0.203	0.111	-8.976
<b>CXCL10<sup>d</sup></b>	<b>Chemokine (C-X-C motif) ligand 10</b>	NM_001565.2	20.125	4.560	0.227	-4.414
EGR1	Early growth response 1	NM_001964.2	0.346	0.078	0.227	-4.413
<b>HES4</b>	<b>Hairy and enhancer of split 4 (<i>Drosophila</i>)</b>	NM_021170.2	6.650	1.761	0.265	-3.776
<b>RSAD2</b>	<b>Radical S-adenosyl methionine domain containing 2</b>	NM_080657.4	25.370	6.750	0.266	-3.758
<b>IFIT1</b>	<b>IFN-induced protein with tetratricopeptide repeats 1</b>	NM_001548.3	18.932	5.058	0.267	-3.743
RNASE6	RNase, RNase A family, k6	NM_005615.4	1.010	0.275	0.272	-3.674
<b>CCL8</b>	<b>Chemokine (C-C motif) ligand 8</b>	NM_005623.2	14.187	3.972	0.280	-3.572
<b>TNFSF10</b>	<b>TNF (ligand) superfamily, member 10</b>	NM_003810.2	6.023	1.750	0.291	-3.442
<b>ISG15</b>	<b>ISG15 ubiquitin-like modifier</b>	NM_005101.1	11.897	3.686	0.310	-3.227
FGL2	Fibrinogen-like 2	NM_006682.1	0.861	0.276	0.321	-3.115
<b>IFIT2</b>	<b>IFN-induced protein with tetratricopeptide repeats 2</b>	NM_001547.4	11.200	3.603	0.322	-3.108
<b>PMP22</b>	<b>Peripheral myelin protein 22</b>	NM_153321.1	1.320	0.449	0.340	-2.940
<b>IFITM3</b>	<b>IFN-induced transmembrane protein 3 (1-8U)</b>	NM_021034.2	5.285	1.861	0.352	-2.839
STAB1	Stabilin 1	NM_015136.2	0.999	0.359	0.359	-2.787
DHRS9	Dehydrogenase/reductase (SDR family) member 9	NM_005771.3	3.093	1.125	0.364	-2.750
<b>IL1R2</b>	<b>IL-1 receptor, type II</b>	NM_173343.1	0.975	0.368	0.377	-2.652
RCBTB2	Regulator of chromosome condensation (RCC1) and BTB (POZ)	NM_001268.2	0.980	0.373	0.381	-2.627
CD300LF	CD300 molecule-like family member f	NM_139018.2	0.838	0.323	0.385	-2.596
<b>OASL</b>	<b>2',5'-oligoadenylate synthetase-like</b>	NM_198213.1	6.569	2.618	0.399	-2.509
<b>CXCL5</b>	<b>Chemokine (C-X-C motif) ligand 5</b>	NM_002994.3	2.968	7.427	2.502	2.502
SPRED1	Sprouty-related, EVH1 domain containing 1	NM_152594.1	0.883	0.362	0.410	-2.440
DDAH2	Dimethylarginine dimethylaminohydrolase 2	NM_013974.1	1.381	0.587	0.425	-2.352
GPR160	G protein-coupled receptor 160	NM_014373.1	1.054	0.452	0.429	-2.334
<b>IFIT3</b>	<b>IFN-induced protein with tetratricopeptide repeats 3</b>	NM_001549.2	15.544	6.675	0.429	-2.329
<b>TNFSF13B</b>	<b>TNF (ligand) superfamily, member 13b</b>	NM_006573.3	2.388	1.030	0.431	-2.318
C6ORF105	Chromosome 6 open reading frame 105	NM_032744.1	1.127	2.564	2.275	2.275
<b>OAS1</b>	<b>2',5'-oligoadenylate synthetase 1, 40/46 kDa</b>	NM_001032409.1	6.404	2.846	0.444	-2.250
<b>CYP27A1</b>	<b>Cytochrome P450, family 27, subfamily A, polypeptide 1</b>	NM_000784.2	0.564	1.264	2.242	2.242
HPSE	Heparanase	NM_006665.3	2.672	1.197	0.448	-2.232
ASGR1	Asialoglycoprotein receptor 1	NM_001671.2	0.451	0.203	0.451	-2.219
LMO2	LIM domain only 2 (rhombotin-like 1)	NM_005574.2	1.324	0.598	0.452	-2.214
<b>DDX58</b>	<b>DEAD (Asp-Glu-Ala-Asp) box polypeptide 58</b>	NM_014314.3	4.491	2.031	0.452	-2.211
FPR3	Formyl peptide receptor 3	NM_002030.3	1.391	0.629	0.453	-2.210
<b>RTP4</b>	<b>Receptor (chemosensory) transporter protein 4</b>	NM_022147.2	4.198	1.916	0.456	-2.191
<b>IL1A</b>	<b>IL-1, <math>\alpha</math> (IL1A)</b>	NM_000575.3	0.635	1.376	2.165	2.165
<b>TGFBI</b>	<b>TGF, <math>\beta</math> induced, 68 kDa</b>	NM_000358.1	1.416	0.662	0.468	-2.139
<b>MERTK</b>	<b>c-mer proto-oncogene tyrosine kinase</b>	NM_006343.2	1.362	0.644	0.473	-2.115
<b>GBP1</b>	<b>Guanylate-binding protein 1, IFN inducible, 67 kDa</b>	NM_002053.1	3.501	1.664	0.475	-2.104
PAICS	Phosphoribosylaminoimidazole carboxylase synthetase	NM_006452.3	0.901	1.894	2.101	2.101
TNFRSF21	TNF receptor superfamily, member 21	NM_014452.3	1.748	0.835	0.478	-2.094
SOD2	Superoxide dismutase 2, mitochondrial	NM_001024466.1	0.961	2.005	2.086	2.086
RNASE2	RNase, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	NM_002934.2	1.392	0.670	0.481	-2.078
<b>LYZ</b>	<b>Lysozyme</b>	NM_000239.1	0.609	0.297	0.488	-2.049
<b>CD86</b>	<b>CD86 Ag (CD28 Ag ligand 2, B7-2 Ag)</b>	NM_006889.3	1.310	0.640	0.488	-2.048
FAM46A	Family with sequence similarity 46, member A	NM_017633.2	2.654	1.303	0.491	-2.037
TNS3	Tensin 3	NM_022748.10	1.282	2.590	2.021	2.021
SLC2A6	Solute carrier family 2 (facilitated glucose transporter), member 6	NM_017585.2	0.752	1.515	2.014	2.014
STS-1	Cbl-interacting protein Sts-1	NM_032873.3	0.960	1.926	2.007	2.007
CCL4L1	Chemokine (C-C motif) ligand 4-like 1	NM_001001435.2	0.685	1.362	1.988	1.988

<sup>a</sup>Mean fold change (FC) from two donors relative to unstimulated controls.<sup>b</sup>Ratio of fold changes from C1q-IC stimulations of PBMCs over ICs alone.<sup>c</sup>Ratio converted to fold change in which positive numbers indicate fold upregulated and negative numbers indicate fold downregulated.<sup>d</sup>Bold lettering indicates type I ISGs.

donors (Fig. 2C, 2D). Of the top 50 genes listed in Table I, the expression of 40 of 50 was reduced by C1q (from 1.5- to 4.4-fold), and only six of these ISGs were not changed. Interestingly, 25 of the top 50 genes regulated by C1q-ICs were not ISGs (Table II). Transcripts encoding multiple cytokines/chemokines (e.g., CCL20, CCL23, CXCL1), receptors (e.g., CD36, STAB1, TNFRSF4, FCGR2A), and enzymes (e.g., RNASE1,2,6, ITK, HPSE, SOD2, SYK) were either upregulated or downregulated in the presence of C1q-ICs, reinforcing the finding that C1q affects multiple pathways that are not direct targets of type I IFNs.

#### *SLE ICs induce a limited inflammatory response in isolated CD14<sup>+</sup> monocytes*

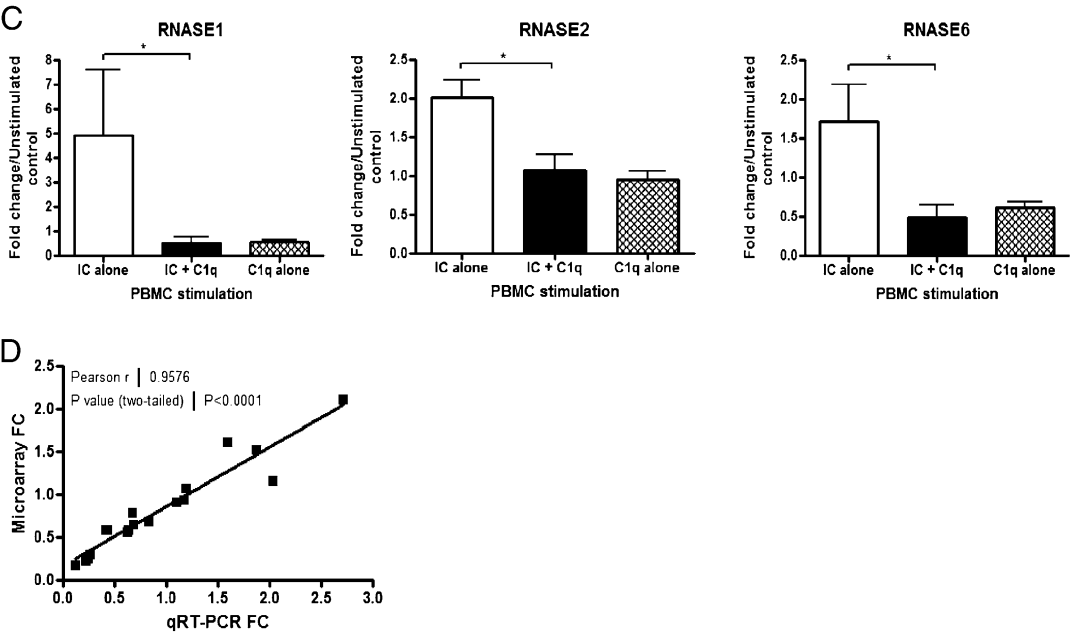
Our results to date indicate that when whole PBMCs are exposed to SLE ICs, the transcriptome reflects a powerful inflammatory response dominated by changes most closely linked to the type I IFN pathway. Yet, ICs are known to engage the activating receptor Fc $\gamma$ RIIA, which is abundantly expressed on CD14<sup>+</sup> monocytes and would be expected to induce other inflammatory cytokines (4, 35, 36). To address the inflammatory potential of SLE ICs directly on monocytes, we isolated CD14<sup>+</sup> monocytes to >95% purity

A Top 5 Canonical Pathways

Name	p-value	Ratio
Interferon signaling	6.79E-07	7/34 (0.206)
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.44E-05	8/84 (0.095)
Activation of IRF by Cytosolic Pattern Recognition Receptors	5.29E-05	7/68 (0.103)
Pathogenesis of Multiple Sclerosis	2.28E-04	3/9 (0.333)
Role of Hypercytokinemia/hyperchemokine in the Pathogenesis of Influenza	5.21E-04	5/46 (0.109)

B Top 5 Networks

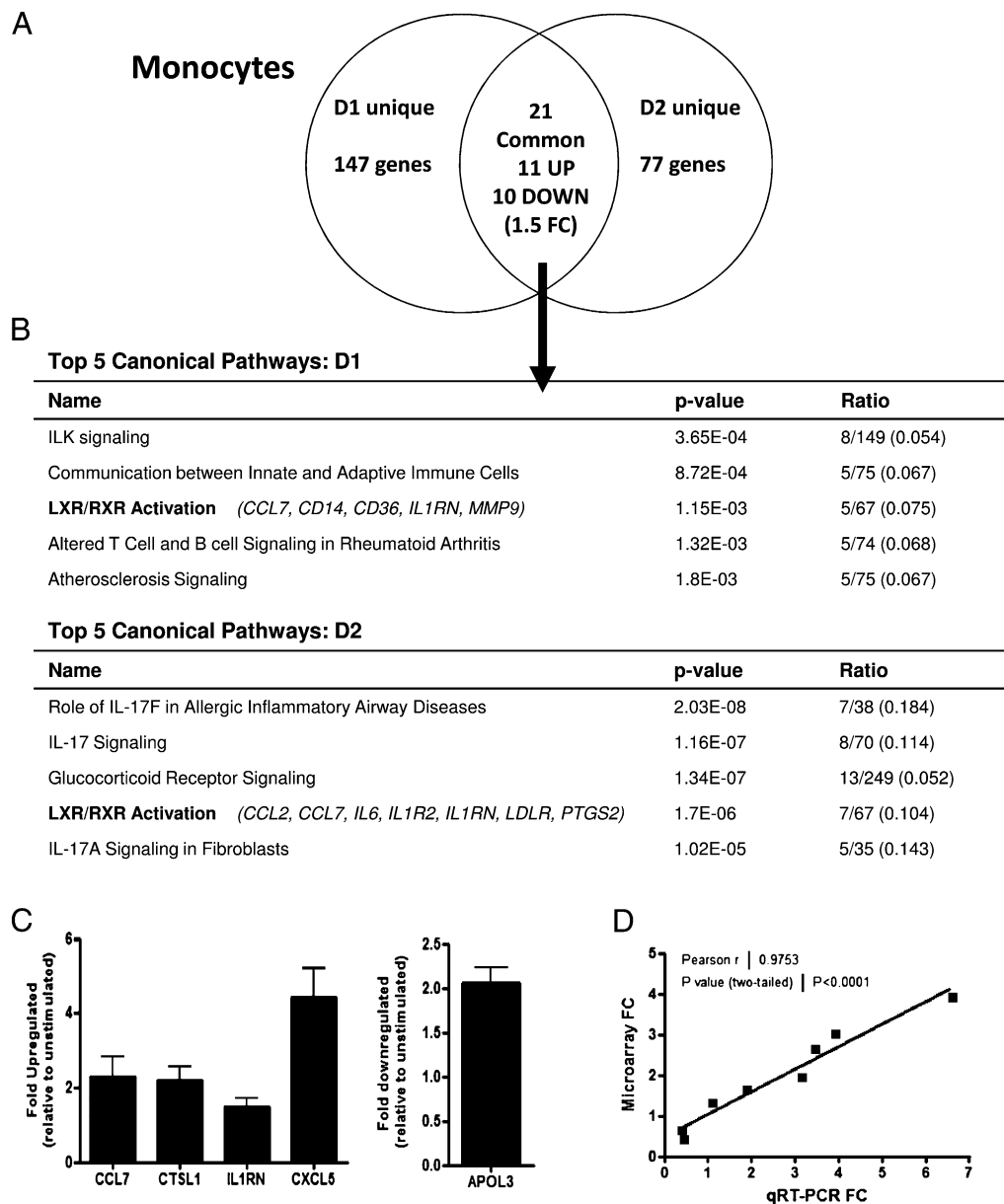
Network
Antimicrobial Response, Inflammatory Response, Infection Mechanism
Antigen Presentation, Cellular Movement, Hematological System Development and Function
Infection Mechanism, Organismal Injury and Abnormalities, Antimicrobial Response
Nucleic Acid Metabolism, Gene Expression, Antimicrobial Response
Antimicrobial Response, RNA damage and Repair, Cellular Movement



**FIGURE 2.** Differential expression of genes and pathways in PBMCs after exposure to C1q-ICs compared with ICs alone. *A* and *B*, SLE ICs were added to the same normal donor PBMCs as in Fig. 1 in the presence or absence of purified C1q (50  $\mu$ g/ml). *A* and *B*, The top five canonical pathways (*A*) and networks (*B*) are shown for the comparison of C1q-ICs to ICs alone. *C*, Three downregulated genes from the microarray data were quantified by qRT-PCR with a total of four independent normal PBMC donor stimulations. Results are shown as fold change compared with unstimulated controls with error bars representing the SEM. *D*, The fold changes from the microarray and qRT-PCR for IC-alone, C1q-IC, and C1q-alone conditions were plotted, and the Pearson correlation coefficient was calculated ( $r = 0.9576$ ,  $p < 0.0001$ ).  $*p < 0.05$  with one-way ANOVA and Tukey's multiple comparison posttest.

with no contaminating pDCs detected and then incubated them with the same SLE ICs and performed microarray expression analysis as for total PBMCs. Surprisingly, compared with whole PBMCs, many fewer genes were modulated similarly in both donors' purified monocytes (150 genes in whole PBMCs versus 21 genes in monocytes, Fig. 3*A*, Table III) with one top canonical pathway in common (Fig. 3*B*). A strong correlation between the microarray and qRT-PCR results was observed with five genes using four different PBMC donors (Fig. 3*C*, 3*D*). Because 168 genes were regulated in D1 and 98 in D2 monocytes, but only 21 genes upregulated and downregulated, respectively, in both donors, there was a much greater degree of individual variation in monocytes from different donors compared with PBMCs. Apart

from the greater degree of variation in monocytes, changes in gene expression were more modest: only 7 and 14 genes were modulated 2-fold or greater in each donor's monocytes (with three genes commonly regulated in both donors) as compared with 121 and 85 modulated 2-fold or greater for each donor's PBMCs (with 68 genes commonly regulated in both donors). The only two transcripts induced 2-fold or greater in both donors' monocytes were solute carrier family 16, member 10 (SLC16A10, also known as monocarboxylate transporter) and CXCL5. SLC16A10 is a member of a family of plasma membrane amino acid transporters that mediate the transport of aromatic amino acids across the plasma membrane. Although 9 of 21 genes regulated by SLE ICs in purified monocytes have previously been linked to type I



**FIGURE 3.** Differential expression of genes and pathways in purified CD14<sup>+</sup> monocytes after exposure to SLE ICs. *A* and *B*, SLE ICs were added to normal donor-purified monocytes for 5 h and RNA isolated for microarray analysis, as described in *Materials and Methods*. *A*, Summary of genes differentially regulated >1.5-fold is shown, with the intersection representing overlapping genes with two different donors (D1 and D2). *B*, The top five canonical pathways for each donor are shown, with only one canonical pathway shared between them (in bold with genes listed in parentheses). *C*, Four upregulated and one downregulated gene from the microarray data were quantified by qRT-PCR with a total of four independent monocyte stimulations. Results are shown as fold upregulated or downregulated comparatively to unstimulated controls. *D*, The fold changes from the microarray and qRT-PCR for the same samples were plotted, and the Pearson correlation coefficient was calculated ( $r = 0.9753$ ,  $p < 0.0001$ ).

IFNs (bolded in Table III), these were expressed at low levels, and the ISG transcripts that were most strongly induced in PBMC, such as RSAD2, CXCL10, IFIT1-3, and OAS1, OASL were not expressed in isolated monocyte cultures exposed to ICs. Of genes differentially expressed, seven were common between PBMCs and purified monocytes with similar levels of expression (SLC16A10, CXCL5, CCL7, CRADD, CTSL1, IL1RN, and TLR8).

#### *C1q modulates monocyte gene expression induced by SLE ICs*

C1q binding to ICs increases binding to monocytes and away from pDCs and also affects intracellular trafficking within monocytes (15). To determine whether the composition of the IC (C1q present or not) affected gene induction as well, we compared gene ex-

pression in isolated monocytes exposed to C1q-ICs versus ICs alone. In total, 36 genes were differentially expressed (Table IV) and 11 genes were concordantly regulated compared with total PBMCs (ADAP2, C7ORF50, CCL20, CCL4L1, H1FX, IL1A, MERTK, PTMA, RCBTB2, SLC39A8, TNFRSF4). The gene TNIP3 (also known as ABIN-3) was upregulated ~2-fold by C1q-ICs and may be related to our findings that C1q is anti-inflammatory as ABIN-3 inhibits NF- $\kappa$ B activation induced by LPS, IL-1, and TNF- $\alpha$  (37, 38). Another GOI that was upregulated ~1.9-fold by C1q-ICs compared with ICs alone was CCL20. One function of CCL20 is to downregulate reactive oxygen species production in monocytes (39), again demonstrating that C1q affects multiple downstream anti-inflammatory pathways independent of IFN- $\alpha$ .



Table III. Genes regulated &gt;1.5-fold upon addition of SLE ICs to purified monocytes

Gene	Gene Name	PROBE_ID <sup>a</sup>	REFSEQ_ID	D1 FC <sup>b</sup>	D2 FC <sup>b</sup>	Mean FC <sup>b</sup>
SLC16A10	Solute carrier family 16, member 10	ILMN_1782938	NM_018593.3	2.667	5.842	4.255
<b>CXCL5<sup>c</sup></b>	<b>Chemokine (C-X-C motif) ligand 5</b>	ILMN_2171384	NM_002994.3	2.651	3.916	3.283
LOC150297	Hypothetical protein LOC150297	ILMN_1711067	NM_001010859.1	-2.074	-3.241	-2.658
<b>CCL7</b>	<b>Chemokine (C-C motif) ligand 7</b>	ILMN_1683456	NM_006273.2	1.648	3.006	2.327
<b>APOL3</b>	<b>Apolipoprotein L, 3</b>	ILMN_1756862	NM_145641.1	-1.569	-2.361	-1.965
<b>CD93</b>	<b>CD93 molecule</b>	ILMN_1704730	NM_012072.3	1.991	1.672	1.832
EGR2	Early growth response 2	ILMN_1743199	NM_000399.2	-2.047	-1.577	-1.812
RAB20	RAB20, member RAS oncogene family	ILMN_1708881	NM_017817.1	1.662	1.956	1.809
<b>CTSL1</b>	<b>Cathepsin L1</b>	ILMN_1694757	NM_001912.3	1.633	1.933	1.783
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	ILMN_1690282	NM_003805.3	1.799	1.755	1.777
TLR8	Toll-like receptor 8	ILMN_1657892	NM_138636.2	-1.621	-1.889	-1.755
<b>BIRC3</b>	<b>Baculoviral IAP repeat-containing 3</b>	ILMN_2405684	NM_182962.1	-1.917	-1.591	-1.754
<b>IL1RN</b>	<b>IL-1R antagonist</b>	ILMN_1689734	NM_173842.1	1.637	1.775	1.706
GK5	Glycerol kinase 5 (putative)	ILMN_1673892	NM_001039547.1	1.546	1.850	1.698
C19ORF59	Chromosome 19 open reading frame 59	ILMN_1762713	NM_174918.2	1.624	1.762	1.693
<b>C21ORF7</b>	<b>Chromosome 21 open reading frame 7</b>	ILMN_1699071	NM_020152.2	-1.648	-1.661	-1.654
FFAR2	Free fatty acid receptor 2	ILMN_1797895	NM_005306.1	-1.773	-1.503	-1.638
STEAP4	STEAP family member 4	ILMN_1772036	NM_024636.2	-1.707	-1.538	-1.623
FKBP5	FK506-binding protein 5	ILMN_1778444	NM_004117.2	-1.550	-1.693	-1.621
<b>HIF1A</b>	<b>Hypoxia-inducible factor 1, <math>\alpha</math> subunit</b>	ILMN_1763260	NM_001530.2	1.501	1.656	1.578
LTB	Lymphotoxin $\beta$ (TNF superfamily, member 3)	ILMN_2376205	NM_002341.1	-1.505	-1.550	-1.528

<sup>a</sup>Probe ID from Illumina Human Ref-8v3 Expression BeadChips.<sup>b</sup>FC, fold change relative to unstimulated controls. D1 and D2, donor 1 and donor 2. Positive numbers indicate fold upregulated; negative numbers indicate fold downregulated.<sup>c</sup>Bold lettering indicates type I ISGs.

#### Differential effects of SLE ICs on monocyte subsets

Microarray analysis revealed a lower magnitude of change of gene expression on isolated CD14<sup>+</sup> monocytes compared with PBMCs. Maturation and activation of monocytes led to an upregulation of costimulatory molecule expression; therefore, we further investigated the question of the inflammatory potential of SLE ICs on monocytes by flow cytometric analysis. In agreement with the limited changes in gene expression observed, we found that exposure of total monocytes to SLE ICs did not induce significant upregulation of CD86 or CD40 (Fig. 4A). As a positive control, the TLR3 agonist poly(I:C) upregulated both CD86 and CD40 4- to 5-fold (data not shown). If IFN- $\alpha$  was the sole factor produced in total PBMC cultures that allowed monocytes to respond to SLE ICs, then priming cells with IFN- $\alpha$  should restore activation by ICs. Whereas IFN- $\alpha$  priming itself upregulated both CD86 and CD40, when we compared costimulatory protein expression in the presence or absence of IFN- $\alpha$ , we observed no differences in CD86 or CD40 upregulation on monocytes when stimulated by three different SLE ICs (Fig. 4B). These data support the gene array findings of limited activation of isolated CD14 monocytes by SLE ICs and that IFN- $\alpha$  itself plays a greater role in costimulatory molecule upregulation on monocytes.

Monocytes are a heterogeneous population of cells that, based on CD14 and CD16 expression, can be divided into three functional subsets, as follows: CD14<sup>dim</sup>CD16<sup>+</sup>, CD14<sup>+</sup>CD16<sup>+</sup>, and CD14<sup>+</sup>CD16<sup>-</sup> (17). Because Cros et al. (17) reported that it is the minor (comprising only ~7% of total monocytes) CD14<sup>dim</sup>CD16<sup>+</sup> patrolling monocyte subpopulation that preferentially responds to TLR7 and eight agonists, we tested whether SLE ICs could upregulate CD86 expression on this rare circulating subset. When monocyte subsets were sorted to high purities and cultured with ICs for 2 d, we found that SLE ICs did in fact upregulate CD86 expression on CD14<sup>dim</sup>, but not the other monocyte subsets (Fig. 4C). When we compared binding of ICs to each subset within total PBMC cultures, we found that whereas all monocyte subsets bound ICs, the greatest binding was to the CD14<sup>+</sup>

CD16<sup>+</sup> subset (Fig. 4D). With addition of C1q, the IC binding to all subsets increased, but binding increased 5- to 6-fold on both of the CD14<sup>high</sup> monocyte subsets, although for the CD14<sup>dim</sup> subset the increase was only 3-fold (Fig. 4D). Taken together, these observations indicate that ICs induce limited inflammatory gene expression or upregulation of costimulatory molecules on total CD14<sup>+</sup> monocytes. They do, however, upregulate CD86 expression on the CD14<sup>dim</sup> population, and C1q preferentially enhances monocyte binding away from the CD14<sup>dim</sup> population.

#### pDCs are required for strong activation of CD14<sup>+</sup> monocytes by SLE ICs

The findings above suggest that most monocytes (the CD14<sup>dim</sup> subset being the exception) are poorly activated in isolation, but are potentially activated in the presence of other blood cells. To determine whether activation of pDCs is specifically required for the inflammatory potential of SLE ICs on monocytes, we compared CD86 and CD40 expression on monocytes in total PBMCs and then again following pDC depletion. As shown in Fig. 5A, monocytes in PBMCs were strongly activated by SLE ICs after 2 d in culture, as measured by upregulation of the expression of CD86 and CD40 by flow cytometry (Fig. 5). Very similar activation of monocytes in PBMCs was observed if SLE ICs were formed with purified SLE IgG instead of using diluted patient serum (Supplemental Fig. 1), and normal serum did not upregulate CD86 or CD40 expression in any stimulation condition (data not shown). When pDCs were depleted from PBMCs such that IFN- $\alpha$  was not detected in response to SLE ICs nor loxoribine (TLR7 agonist), monocytes were no longer potentially activated by SLE ICs as determined by significantly lower levels of CD86 and CD40 (Fig. 5B).

Consistent with these findings, we observed that SLE ICs induced high levels of cytokines and chemokines known to be produced by monocytes such as IL-1RA (same as IL-1RN in our arrays), IP-10 (CXCL10), and MCP-1 in total PBMCs, but not in pDC-depleted cultures (Fig. 6A). MCP-3 induction varied depending on the donor used, and IL-8 induction was not depen-

Table IV. Genes regulated &gt;1.5-fold upon addition of C1q to SLE ICs in purified monocyte cultures

Gene	Gene Name	REFSEQ_ID	Mean IC FC <sup>a</sup>	Mean C1q-IC FC <sup>a</sup>	C1q-IC/IC Ratio <sup>b</sup>	C1q-IC/IC FC <sup>c</sup>
C7ORF50	Chromosome 7 open reading frame 50	NM_032350.4	0.790	0.392	0.496	-2.015
<b>TNIP3<sup>d</sup></b>	<b>TNFAIP3-interacting protein 3</b>	NM_024873.3	1.208	2.393	1.980	1.980
<b>MERTK</b>	<b>c-mer proto-oncogene tyrosine kinase</b>	NM_006343.2	1.573	0.811	0.516	-1.939
CCL20	Chemokine (C-C motif) ligand 20	NM_004591.1	1.160	2.230	1.923	1.923
SLC39A8	Solute carrier family 39 (zinc transporter), member 8	NM_022154.5	1.020	1.890	1.853	1.853
IL6	IL-6 (IFN, $\beta$ 2)	NM_000600.1	1.520	2.635	1.734	1.734
TNFRSF4	TNF receptor superfamily, member 4	NM_003327.2	0.739	1.279	1.730	1.730
EREG	Epregrulin	NM_001432.2	1.015	1.715	1.689	1.689
<b>CXCL10</b>	<b>Chemokine (C-X-C motif) ligand 10</b>	NM_001565.2	0.680	1.146	1.684	1.684
<b>GCH1</b>	<b>GTP cyclohydrolase 1</b>	NM_001024070.1	0.893	1.438	1.610	1.610
<b>IL1A</b>	<b>IL-1, <math>\alpha</math></b>	NM_000575.3	0.985	1.580	1.604	1.604
<b>PLAC8</b>	<b>Placenta-specific 8</b>	NM_016619.1	0.640	1.025	1.602	1.602
FEZ1	Fasciculation and elongation protein $\zeta$ 1 (zyglin I)	NM_005103.3	0.675	1.080	1.601	1.601
ACP5	Acid phosphatase 5, tartrate resistant	NM_001611.2	1.156	0.724	0.627	-1.596
<b>CXCR4</b>	<b>Chemokine (C-X-C motif) receptor 4</b>	NM_001008540.1	1.450	0.910	0.628	-1.593
RGS12	Regulator of G protein signaling 12	NM_002926.3	1.067	0.674	0.632	-1.583
IL17RD	IL-17 receptor D	NM_001080973.1	0.710	1.123	1.581	1.581
RCBTB2	Regulator of chrom condensation (RCC1)	NM_001268.2	1.095	0.697	0.637	-1.571
G0S2	G $\alpha$ /G $\beta$ switch 2	NM_015714.2	1.059	1.654	1.562	1.562
SERPINA1	Serpin peptidase inhibitor, clade A	NM_001002236.1	0.942	1.470	1.561	1.561
<b>IFIT3</b>	<b>IFN-induced protein with tetratricopeptide repeats 3</b>	NM_001549.2	0.870	1.354	1.557	1.557
CCL4L1	Chemokine (C-C motif) ligand 4-like 1	NM_001001435.2	0.840	1.307	1.555	1.555
ODC1	Ornithine decarboxylase 1	NM_002539.1	0.879	0.567	0.645	-1.551
<b>CASP4</b>	<b>Caspase 4, apoptosis-related cysteine peptidase</b>	NM_033306.2	0.993	1.538	1.549	1.549
AK3L1	Adenylate kinase 3-like 1	NM_013410.2	0.852	1.315	1.543	1.543
LOC150297	Hypothetical protein LOC150297	NM_001010859.1	0.395	0.609	1.540	1.540
KLIF	Chemokine-like factor	NM_001040138.1	1.490	0.969	0.650	-1.539
GRAMD1A	GRAM domain containing 1A	NM_020895.2	0.755	1.160	1.537	1.537
RPL9	Ribosomal protein L9 (RPL9), transcript variant 2	NM_001024921.2	0.744	1.140	1.531	1.531
<b>HSPA1A</b>	<b>Heat shock 70-kDa protein 1A (HSPA1A)</b>	NM_005345.4	0.872	1.323	1.517	1.517
<b>CCR7</b>	<b>Chemokine (C-C motif) receptor 7</b>	NM_001838.2	1.000	1.511	1.511	1.511
TM6SF1	Transmembrane 6 superfamily member 1	NM_023003.2	1.005	0.665	0.662	-1.511
ADAP2	ArfGAP with dual PH domains 2	NM_018404.2	1.004	0.665	0.663	-1.509
H1FX	[ <sup>1</sup> H]Histone family, member X	NM_006026.2	0.873	0.581	0.666	-1.502
PTMA	Prothymosin, $\alpha$ (PTMA), transcript variant 1	NM_001099285.1	0.847	1.272	1.502	1.502
<b>EMR1</b>	<b>egf-like module-containing, mucin-like, hormone receptor-like 1</b>	NM_001974.3	0.847	1.271	1.501	1.501

<sup>a</sup>Mean fold change (FC) from two donors relative to unstimulated controls.<sup>b</sup>Ratio of fold changes from C1q-IC stimulations of PBMCs over ICs alone.<sup>c</sup>Ratio converted to fold change in which positive numbers indicate fold upregulated and negative numbers indicate fold downregulated.<sup>d</sup>Bold lettering indicates type I ISGs.

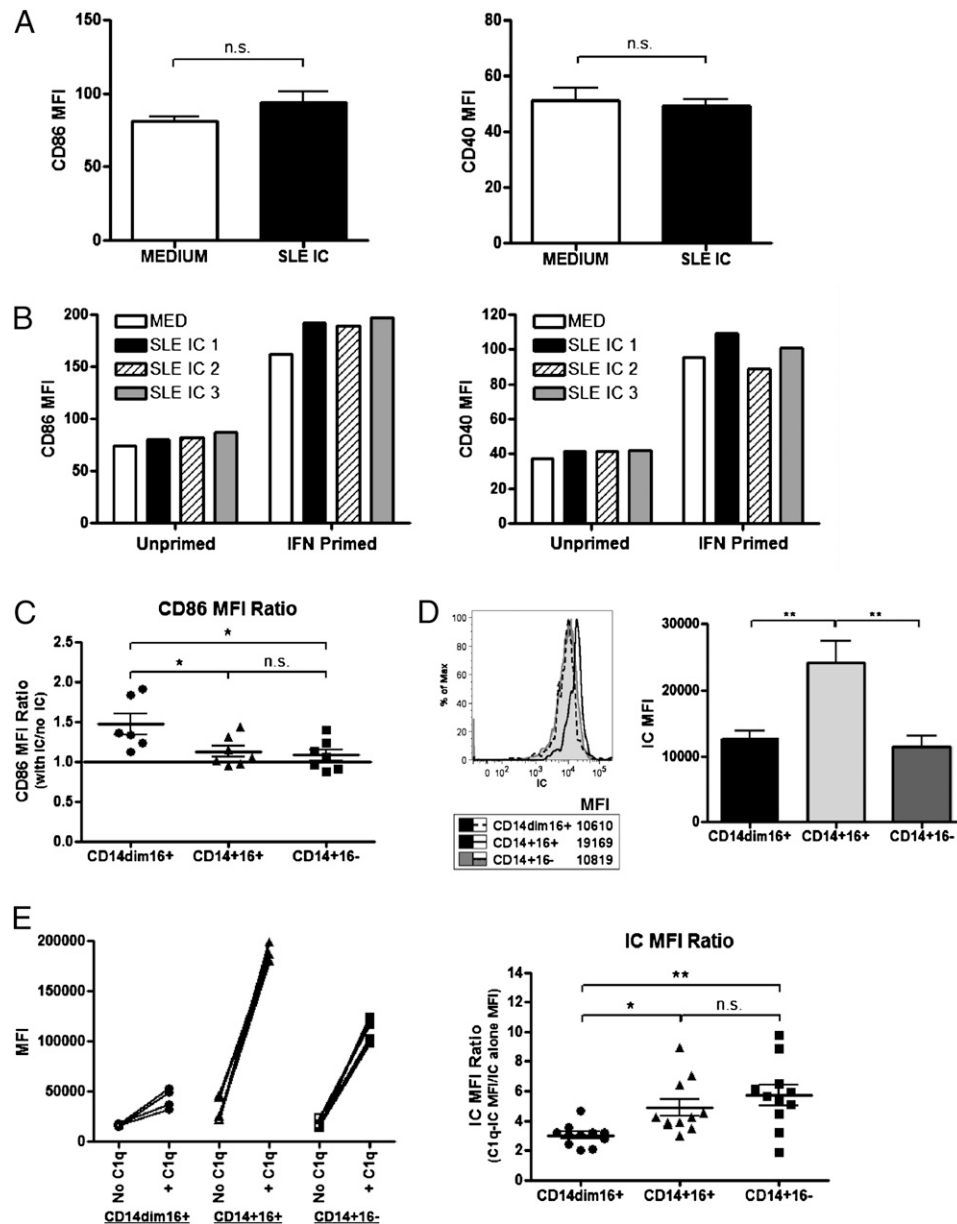
dent on the presence of pDCs, as similar levels were detected in both mock- and pDC-depleted cultures (Fig. 6). Using at least three different ICs for the multiplex and >20 different ICs for TNF- $\alpha$  stimulation in total PBMCs, the ICs did not induce IL-1 $\beta$ , IL-12p40, IL-10, MIP-1 $\alpha$ , or TNF- $\alpha$  compared with unstimulated control cultures (data not shown). Similar to PBMC cultures, purified monocytes produced low levels of MCP-1 (average of 60 pg/ml above background) and, on average, 1259 pg/ml IL-8 above background when stimulated by SLE ICs (data not shown, Fig. 6B). There was no induction of IP-10, IL-1RA, MCP-3, MIP-1 $\alpha$ , IL-12p40, IL-10, TNF- $\alpha$ , or IL-1 $\beta$  above background when ICs were added to purified monocytes (data not shown). Despite a small (~1.7-fold) increase in IL-6 mRNA expression seen in the microarray following C1q addition to ICs, this increase was not confirmed by ELISA (data not shown, four independent experiments).

Although pDCs were required for maximal expression of costimulatory molecule expression by monocytes, the addition of neutralizing Ab to IFN- $\alpha$  either had minimal effect or reduced CD86 and CD40 expression at most 1.6- and 1.4-fold, respectively, indicating that other factors in addition to IFN- $\alpha$  are important for monocyte activation by ICs. Together our data suggest pDCs are required for SLE ICs to efficiently stimulate the major monocyte populations (CD14<sup>+</sup>CD16<sup>-</sup> or CD14<sup>+</sup>CD16<sup>+</sup>), whereas the rare subset of CD14<sup>dim</sup> monocytes is able to respond directly to SLE ICs.

## Discussion

The goals of this study were several fold. We wished to identify common changes in gene expression by SLE ICs in total PBMCs, to identify changes in genes or pathways when C1q was present in ICs, and to determine the extent to which SLE ICs induced common inflammatory pathways directly on monocytes. Studies were therefore performed with at least two SLE ICs and at least two different normal donor cells. The results of these studies reveal that pDCs and CD14<sup>dim</sup> monocytes are key targets of SLE ICs, that C1q influences IC activation of both of these cells, and we identify multiple genes that are regulated depending upon the target cells and composition of ICs.

Experimentally, it has been shown that ICs can induce TNF- $\alpha$  (40–42) or, more recently, IFN- $\alpha$  (33). In our study, addition of SLE ICs to unprimed normal PBMCs induced a dominant type I IFN pattern of gene transcription with little or no TNF- $\alpha$  production, consistent with the ex vivo gene signature in SLE (43, 44). When we compared genes upregulated in normal PBMCs by SLE ICs to published SLE patient peripheral blood ex vivo transcript profiles (45), we observed that 76 of the 150 genes regulated by in vitro IC exposure were represented in SLE patients' PBMC transcriptome. These findings are therefore consistent with the idea that circulating ICs account to a significant degree for the IFN signature observed in SLE patients' blood cells. The lack of full

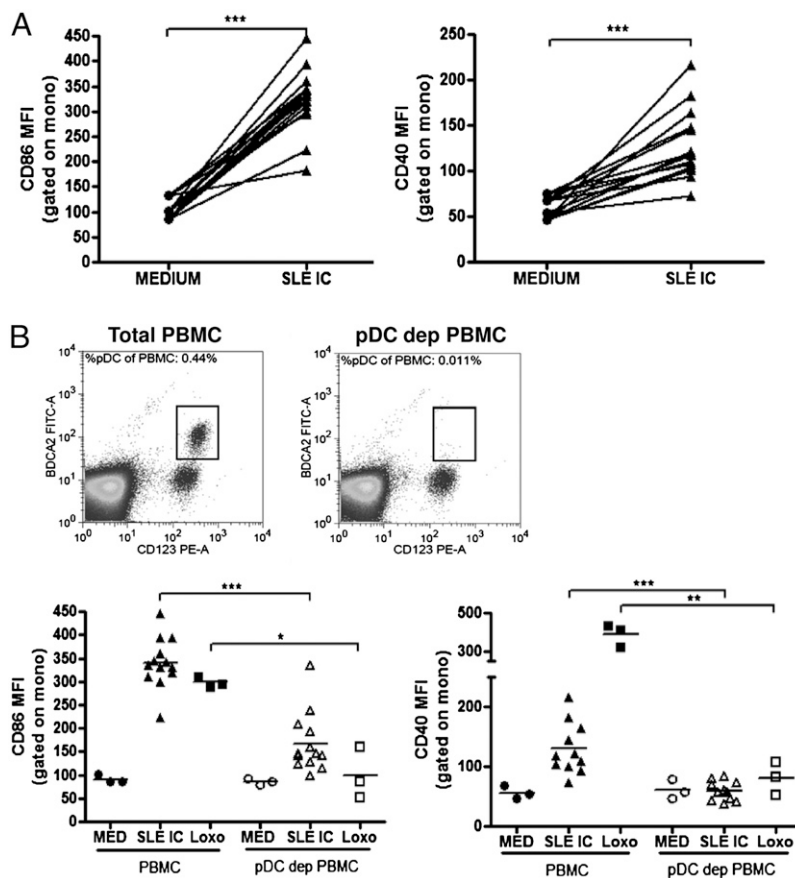


**FIGURE 4.** SLE ICs upregulate CD86 only on CD14<sup>dim</sup> monocytes. *A* and *B*, SLE ICs were incubated with total CD14<sup>+</sup> purified monocytes (>96% pure) in medium alone (*A*) or with or without 4-h priming with 500 U/ml type I IFN (*B*). After 2 d in culture, CD86 and CD40 expression was quantified by flow cytometry. Results in *A* are expressed as MFI from three independent experiments with two to three different SLE patient ICs per experiment. Results in *B* are representative of three independent experiments with three different SLE patient ICs added per experiment. *C*, The three different monocyte subsets, CD14<sup>dim</sup>CD16<sup>+</sup>, CD14<sup>+</sup>CD16<sup>+</sup>, and CD14<sup>+</sup>CD16<sup>-</sup>, were isolated by flow sorting and exposed to SLE ICs, as in *A*. CD86 expression was quantified and expressed as the ratio of MFI following IC stimulation/medium (no IC) control. *D* and *E*, SLE ICs were formed using fluorescently labeled SmRNP Ag in the absence (*D*) or presence of C1q (20  $\mu$ g/ml) (*E*) and allowed to bind to PBMCs for 30 min on ice. *D*, *Left panel*, Representative histogram with the MFI of ICs bound to each monocyte subset shown in the legend below. *Right panel*, Compiled MFI data of SLE IC binding to each monocyte subset with one to six SLE ICs per experiment for a total of three independent experiments. *E*, *Left panel*, Representative experiment with four SLE ICs and the binding to monocyte subsets within PBMCs with or without C1q. *Right panel*, Compiled data for SLE IC binding to monocyte subsets expressed as a MFI ratio of C1q-IC to IC binding in the absence of C1q (IC alone) from three experiments with a total of 11 SLE patient ICs. Horizontal lines are mean  $\pm$  SEM. n.s., not significant; \* $p$  < 0.05, \*\* $p$  < 0.01 with unpaired *t* test (*A*) and one-way ANOVA with Tukey's multiple comparison posttest (*C–E*).

concordance could be explained by the acute nature of in vitro stimulation versus chronic stimulation in vivo. In addition, certain autoantigens released during apoptosis may not be present in our freeze-thawed extract to make ICs. Another possible explanation is that DNA released from neutrophil nets can form ICs and directly stimulate TLR9 in pDCs, but neutrophils were not added in our PBMC cultures (46, 47). Whether other potent IFN inducers such as endogenous (48) or exogenous (49) virus infection contribute to type I IFN stimulation as well remains to be determined.

ICs induced the expression of multiple genes and pathways that are implicated in the pathogenesis of SLE. For example, upregulation of nucleic acid receptors and associated adaptor molecules that are known ISGs (DDX58 [RIG-I], TRIM25, UNC93B1, IRF7, IFIH1 [MDA5], MyD88) would serve as a positive feedback loop to enhance responses to ICs or other nucleic acid stimuli in SLE. SLE ICs also upregulated the expression of two recently identified anti-inflammatory molecules that are not known to be regulated by IFN- $\alpha$ : IL1F7 (also known as IL-37) 5-fold and IL-4-induced

**FIGURE 5.** SLE ICs upregulate monocyte CD86 and CD40 expression in PBMC cultures, but increased expression requires the presence of pDCs. SLE ICs were incubated with total PBMC cultures, as in Fig. 1. A, After 2 d, CD14<sup>+</sup> monocytes were analyzed for CD86 and CD40 expression by flow cytometry. Results from four independent experiments with three to five different SLE patient ICs per experiment are expressed as MFI in which each line is a different SLE IC stimulation. B, Monocyte expression of CD86 and CD40 following SLE IC or loxoribine (200  $\mu$ M) stimulation was analyzed by flow cytometry, as in A, except that PBMCs were either mock depleted or depleted of pDCs (pDC dep) with BDCA-4 magnetic beads (representative depletion shown in upper panel). MFI results from three independent experiments with at least two different SLE patient ICs per experiment are shown in the lower panel. Loxo, loxoribine. MED, medium. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  with unpaired  $t$  test (A) and paired  $t$  test (B).

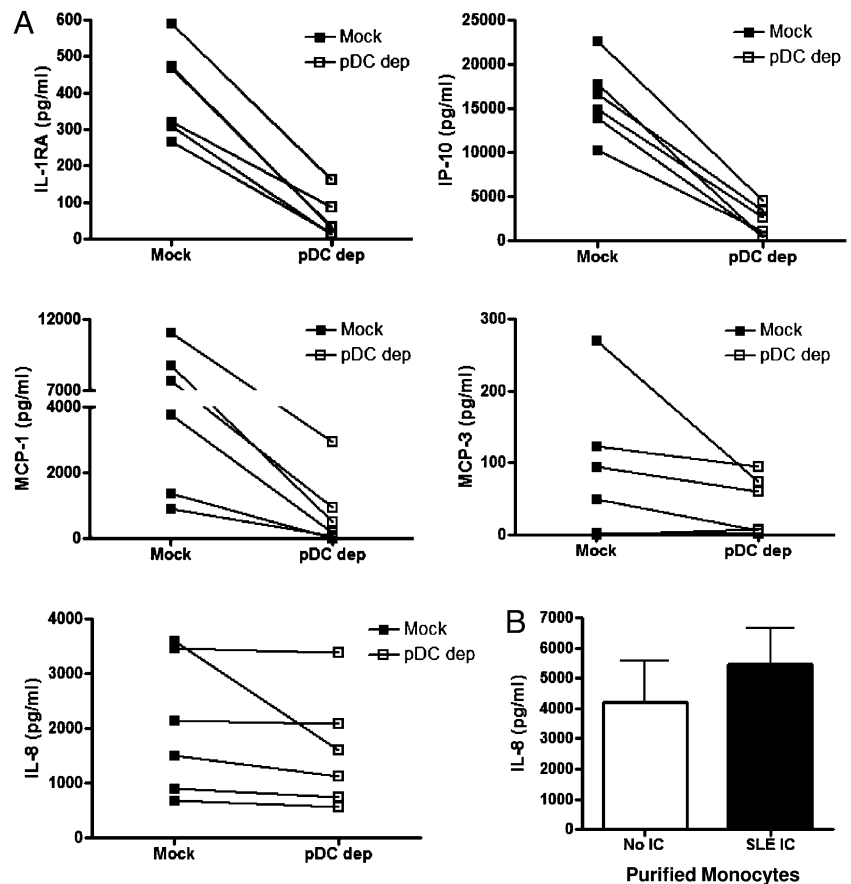


gene 1 (IL4I1) 2-fold. IL-37 exerts an anti-inflammatory effect in response to LPS stimulation, and small interfering RNA knock-down led to enhanced production of multiple proinflammatory cytokines (50). IL4I1 inhibits T cell proliferation and an optimal Th1 response by inhibiting IFN- $\gamma$  and other inflammatory cytokines (51, 52). These genes may therefore serve in a negative feedback loop, and their function would be well worth exploring in SLE patients. Other IC-induced genes (PPARG, ~2-fold and RASGRP3, 1.8-fold) that are not known to be IFN inducible have been implicated in lupus susceptibility or pathogenesis. IC stimulation could potentially explain peroxisome proliferator-activated receptor  $\gamma$  elevation in patients with active SLE (53). RASGRP was recently discovered to be a SLE susceptibility gene associated with the development of malar rash, discoid rash, and antinuclear Ab positivity (54, 55).

Consistent with previously published results on IFN- $\alpha$  suppression by C1q-ICs (14, 15), addition of C1q to ICs resulted in a marked suppression of ISGs and multiple other genes. In total, 81 previously identified type I IFN response genes were downregulated by C1q-ICs compared with ICs alone. Two prominent gene families whose expression was reduced were TNF family members and RNASEs. TNFSF13B (BAFF) and TNFSF10 (TRAIL) have been implicated in SLE (56, 57), and the expression of TNFRSF21 (DR6) impacts B and T cell responses (58). Reduction in expression of these genes by C1q would therefore be predicted to be beneficial in SLE patients. In contrast, RNases are less well studied. RNASE1 (the homolog of bovine RNase A) is expressed in the pancreas, endothelial cells (59), as well as immune cells such as activated monocytes (60). Of interest, RNASE1 secretion was shown to increase in response to extracellular RNA (but not DNA), and addition of RNase 1 or RNase 2 (also known as eosinophil-derived neurotoxin) induced activation of human

monocyte-derived dendritic cells in vitro (61). Because we observed an increase in RNASE1 gene expression with ICs alone, PBMCs may respond to exposure to RNA containing ICs by increasing RNase expression. Increased expression of RNASE2 has been observed previously in expression arrays in SLE patient PBMCs (43, 44) and also noted to be upregulated in inflammatory bowel disease and rheumatoid arthritis patient PBMCs (62, 63). RNASE6 has 47% amino acid sequence similarity to RNASE2 (64) and is most highly expressed in pDCs, monocytes, neutrophils, and B cells (64–66). Because RNASE6 was modestly upregulated by SLE ICs and strongly downregulated by C1q, future studies will be required to determine the role of this protein in the immune response. Because none of the RNASE genes were regulated in purified monocyte cultures, induction of transcription is most likely controlled by inflammatory cytokines released by other immune cell types. Genes that were upregulated by C1q-ICs, such as the antioxidant, SOD2, could also be important in the anti-inflammatory response. Whereas our study has focused on C1q, C4 and C2 deficiency is also associated with increased risk for the development of SLE, although the risk is not as high (reviewed in Ref. 67). Preliminary data suggested that C2 was not required for inhibition of IFN- $\alpha$  induced by SLE ICs (15), and purified C4 protein did not inhibit IC stimulation (data not shown). In vivo it is likely that the early classical complement proteins, C1q, C2, and C4, may act in a common pathway leading to the assembly of C3bi on apoptotic cells, which we, and others, previously showed is important for the clearance of dying cells (8, 9). Because C1q deficiency is associated with a much higher penetrance of SLE, additional immune regulation effects, as we have shown in this study, plus the importance of C1q in IC and apoptotic cell clearance, are most likely involved in disease pathogenesis.





**FIGURE 6.** pDCs are required for maximal induction IL-1RA, IP-10, MCP-1, and MCP-3, but not IL-8, in response to SLE ICs. *A* and *B*, Cytokines/Chemokines were measured by multiplex analysis, as described in *Materials and Methods*, after mock-depleted (Mock) or pDC-depleted (pDC dep) PBMCs (*A*) or purified monocytes (*B*) were exposed to SLE ICs for 2 d of stimulation. Raw data in pg/ml are plotted from two independent experiments with two different donors and three (*A*) or two (*B*) different SLE ICs per experiment. No IC indicates the presence of freeze-thawed Ag alone.

Surprisingly, IC stimulation of purified total CD14<sup>+</sup> monocytes resulted in lower changes in gene expression compared with PBMCs and little to no upregulation of CD40 or CD86. Only the monocarboxylate transporter, SLC16A10, and the neutrophil-attracting chemokine, CXCL5, were induced 2-fold or greater in both donors' monocytes. Although expression of other monocarboxylate transporters has been studied in monocytes (68), little is known of the function of SLC16A10 in these cells. CXCL5, the ligand for the chemokine receptor CXCR2, stimulates the chemotaxis of neutrophils, and therefore can be implicated in the inflammatory properties of SLE ICs on monocytes. The low gene induction by SLE ICs on isolated CD14<sup>+</sup> monocytes is consistent with the finding that pDC depletion of PBMCs resulted in very limited stimulation of monocytes, as determined by costimulatory molecule expression. This may be explained by the much lower expression of TLR7 in monocytes as compared with pDCs (69). Experiments utilizing either addition of type I IFN to monocyte cultures as well as IFN- $\alpha$  neutralization in PBMCs suggested that type I IFN as well as other factors were required for full monocyte activation. A similar requirement for pDCs for stimulation of monocytes was shown by Hornung et al. (69) with the TLR9 agonist CpG. In addition, human B cells require the presence of pDCs and IFN- $\alpha$  for maximal stimulation by TLR7 agonists (70).

ICs are considered to be potent activators of inflammatory responses in Fc $\gamma$ RII-bearing cells of the myeloid lineage associated with the production of TNF- $\alpha$  (35, 36, 40, 41). Several explanations for the differences between our studies compared with some prior studies of the activating effect of ICs on whole monocytes are possible. Most used heat-aggregated IgG or tetanus toxoid as ICs and/or did not exclude endotoxin contamination as a possible cause of TNF- $\alpha$  stimulation. ICs may also have different effects in tissues in vivo. In this context, Geissmann and

colleagues (17) recently identified a rare monocyte subset that in vivo is thought to patrol endothelial surfaces, where they respond rapidly to microbial stimuli. When experiments were performed to examine differences in the responses of the three monocyte subsets, we observed that the CD14<sup>dim</sup> population did appear to be more responsive to SLE ICs as determined by upregulation of CD86 expression, although the maximum TNF- $\alpha$  production was very low (0–60 pg/ml in three experiments, data not shown). Perhaps of further relevance to protection from inflammation (15), the presence of C1q in ICs favored the binding of ICs away from the CD14<sup>dim</sup> population to CD14<sup>high</sup> monocytes.

Of considerable interest, differences were noted in the response to SLE ICs between two different normal monocyte donors. It will be interesting to examine monocyte gene stimulation in a larger number of normal individuals as well as SLE patients in remission to see whether stable patterns are observed and whether patients can be segregated based on the response. Although we confirmed some chemokine mRNA expression changes by multiplex analysis, further work is needed to confirm changes in gene expression at the protein level. In addition, our studies have focused on IC responses in PBMCs. Future experiments to test the physiological consequences of C3b-coated IC handling in whole blood where RBCs and neutrophils could play important roles will be important to determine (47, 71). In summary, these studies identify pDCs and CD14<sup>dim</sup> monocytes as the most important cell targets for SLE ICs and define genes and pathways stimulated in total PBMCs. This information will help to identify additional biomarkers and to design targeted therapies for SLE.

## Acknowledgments

We thank Edward Clark, Grant Hughes for helpful discussion, Thomas Möller for assistance with cytokine multiplex analysis, and Ryan Basom,



Alyssa Dawson, and Jeff Delrow at the Fred Hutchinson Cancer Research Center's Genomics Resource for microarray setup and analysis.

## Disclosures

The authors have no financial conflicts of interest.

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