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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,† Pradipta Ghosh,† and Keith B. Elkon*†

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
dim 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
dim 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
dim (“patrolling”) subpopulation was more responsive to ICs. These observations demonstrate the importance of plasmacytoid dendritic cells, CD14
dim 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: 000–000.

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 FcyRs 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 complement 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
dim 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

Recagents

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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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 murine monoclonal Abs described previously (17, 18). Briefly, cells were stained with the following fluorescently labeled Abs (all from BioLegend, unless otherwise noted): CD14-PE (clone HB19), CD356-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+; this was further divided into three monocyte subsets that included the CD14+ CD16-, CD14+CD16+, and CD14dim/CD16- subsets that were sorted and collected live using a FACSaria 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 autoantigens, and freeze-thawed U937 cells were used as autotaxin, as described previously (15, 18, 19). Briefly, U937 cells (5 × 10⁶/ml) were cultured in 24-well plates at 2 × 10⁶ monocytes per condition from each of two independent experiments, 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 >90% myelomonocytic, and contaminating e-Mycoplasma PCR detection kit (nIRON 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 × 10⁶/well in one well and left unstimulated or stimulated with SLE ICs formed, as above, with or without Clq for 3–6 h. For monocyte stimulation, a total of 1 × 10⁶ monocytes per condition (100 µl) were 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, FcyRia, and TLK7 related (19). Although many SLE patient sera were used in the course of this study, the sera used to make ICs for the microarray experiments both had the following autoantibody profile: Sm, RNP*, Ro-, La-, dsDNA+. ICs were added to normal PBMCs (5 × 10⁷/well) and left unprimed or primed with type 1 IFN and CM-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 >90% myelomonocytic, and contaminating e-Mycoplasma PCR detection kit (nIRON Biotechnology). Toxicity of added inhibitors was monitored by flow cytometry with LIVE/DEAD I/R (Invitrogen).

Microarray data analysis

Probe-level results were generated in GenomeStudio Data Analysis Software’s Gene Expression Module (GSGX) Version 1.5.4 (Illumina). BeadChips results were background corrected and quantile normalized using the Bioconductors 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 Ingenuity 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 not to be 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-ΔΔCT method with unstimulated cells as baseline to determine fold changes for each GOI. Primer sequences for genes are shown in Supplemental Table I.

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; Biologend) and CD40 (clone SC3; 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 mediators 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.
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-α 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). A strong correlation between the microarray and qRT-PCR was shown (Fig. 2B). A strong correlation between the microarray and qRT-PCR results was observed with three genes and four different PBMC

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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+ 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 FcγRIIA, which is abundantly expressed on CD14+ monocytes. The finding that C1q affects multiple pathways that are not direct targets of type I IFN results in a limited inflammatory response in isolated CD14+ monocytes.
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. 3A, Table III) with one top canonical pathway in common (Fig. 3B). A strong correlation between the microarray and qRT-PCR results was observed with five genes using four different PBMC donors (Fig. 3C, 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
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 expression 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 TNIPI3 (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-κB activation induced by LPS, IL-1, and TNF-α (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-α.

![FIGURE 3. Differential expression of genes and pathways in purified CD14+ 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).](http://www.jimmunol.org/)

- **FIGURE 3.** Differential expression of genes and pathways in purified CD14+ 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).
Differential effects of SLE ICs on monocyte subsets

Microarray analysis revealed a lower magnitude of change in gene expression on isolated CD14+ 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 monocytes by flow cytometric analysis. In agreement with the investigated the question of the inflammatory potential of SLE ICs on 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

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

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^aProbe ID from Illumina Human Ref-8v3 Expression BeadChips.
^bFC, fold change relative to unstimulated controls. D1 and D2, donor 1 and donor 2. Positive numbers indicate fold upregulated; negative numbers indicate fold downregulated.
^cBold lettering indicates type I ISGs.

pDCs are required for strong activation of CD14+ monocytes by SLE ICs

The findings above suggest that most monocytes (the CD14dim subset) respond to SLE ICs in- fluently 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 activation of pDCs 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-α was not detected in response to SLE ICs nor loxoribine (TLR7 agonist), monocytes were no longer potently 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 (CCL10), 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-
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-α stimulation in total PBMCs, the ICs did not induce IL-1β, IL-12p40, IL-10, MIP-1α, or TNF-α 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-1α, IL-12p40, IL-10, TNF-α, or IL-1β 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 co-stimulatory molecule expression by monocytes, the addition of neutralizing Ab to IFN-α 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-α 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+CD16− or CD14+CD16+), whereas the rare subset of CD14dim 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 CD14dim 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-α (40–42) or, more recently, IFN-α (33). In our study, addition of SLE ICs to unprimed normal PBMCs induced a dominant type I IFN response (40–42), whereas the addition of IFN-α to normal PBMCs induced a type II IFN response (33). In our study, addition of SLE ICs to unprimed normal PBMCs induced a dominant type I IFN response (40–42), whereas the addition of IFN-α to normal PBMCs induced a type II IFN response (33).
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, IFIHI [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-α: IL1F7 (also known as IL-37) 5-fold and IL-4-induced

**FIGURE 4.** SLE ICs upregulate CD86 only on CD14dim monocytes. A and B, SLE ICs were incubated with total CD14+ 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, CD14dimCD16+, CD14+CD16+, and CD14+CD16−, 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 μg/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 ± 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).
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). IL4II inhibits T cell proliferation and an optimal Th1 response by inhibiting IFN-γ 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 γ 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-α 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 lupus susceptibility or pathogenesis. IC stimulation could potentially explain peroxisome proliferator-activated receptor γ elevation in patients with active SLE (53). RASGRP2 was recently discovered to be a SLE susceptibility gene associated with the development of malar rash, discoid rash, and antinuclear Ab positivity (54, 55).

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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+ 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. A, Monocyte expression of CD86 and CD40 following SLE IC or loxoribine (200 μM) 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).
Surprisingly, IC stimulation of purified total CD14+ 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+ 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-α 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-α for maximal stimulation by TLR7 agonists (70).

ICs are considered to be potent activators of inflammatory responses in FcγRII-bearing cells of the myeloid lineage associated with the production of TNF-α (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-α 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 CD14dim population did appear to be more responsive to SLE ICs as determined by up-regulation of CD86 expression, although the maximum TNF-α 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 CD14dim population to CD14high 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 CD14dim 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.

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References


