

Activation of plasmacytoid dendritic cells and B cells with two structurally different Toll-like receptor 7 agonists

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Abstract

Synthetic Toll-like receptor (TLR) 7 agonists have been suggested as immune modulators in a range of conditions. In contrast, self-derived TLR7 activators, such as RNA-containing immune complexes (RNA-IC), can contribute to autoimmune diseases due to endogenous immune activation. The exact difference in immune cell response between synthetic and endogenous TLR7 triggers is only partly known. An understanding of these differences could aid in the development of new therapeutic agents and provide insights into autoimmune disease mechanisms. We therefore compared the stimulatory capacity of two TLR7 agonists, RNA-IC and a synthetic small molecule DSR-6434, on blood leucocytes, plasmacytoid dendritic cells (pDCs) and B cells from healthy individuals. IFN- α , IL-6, IL-8 and TNF levels were measured by immunoassays, and gene expression in pDCs was analysed by an expression array. DSR-6434 triggered 20-fold lower levels of IFN- α by pDCs, but higher production of IL-6, IL-8 and TNF, compared to RNA-IC. Furthermore, IFN- α and TNF production were increased with exogenous IFN- α 2b priming, whereas IL-8 synthesis by B cells was reduced for both stimuli. Cocultivation of pDCs and B cells increased the RNA-IC-stimulated IFN- α and TNF levels, while only IL-6 production was enhanced in the DSR-6434-stimulated cocultures. When comparing pDCs stimulated with RNA-IC and DSR-6434, twelve genes were differentially expressed (\log_2 fold change >2 , adjusted P -value $<.05$). In conclusion, RNA-IC, which mimics an endogenous TLR7 stimulator, and the synthetic TLR7 agonist DSR-6434 trigger distinct inflammatory profiles in immune cells. This demonstrates the importance of using relevant stimuli when targeting the TLR7 pathway for therapeutic purposes.

1 | INTRODUCTION

Activated plasmacytoid dendritic cells (pDCs) and B cells are two efficient immune regulators that interact in the interplay

between innate and adaptive immunity.¹⁻⁴ Pattern recognition receptors (PRRs) are expressed by various immune cells to sense conserved pathogen-associated molecular patterns (PAMPs) on microorganisms and alert the immune system

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of a possible threat.^{5,6} One important PRR family consists of Toll-like receptors 1 to 10 (TLR1-10), which upon recognition of PAMPs can evoke a powerful inflammatory response.⁶ The pDCs and B cells uniquely express TLR7 and TLR9 in their endo-lysosomal compartments where they sense single-stranded (ss) RNA and double-stranded DNA, respectively.⁷⁻¹⁰ In addition to natural agonists, TLR7 can be activated by synthetic compounds such as the imidazoquinolines resiquimod and imiquimod structurally resembling nucleic acids or by oxoadenine derivatives.¹⁰⁻¹³

The endosomal TLRs are normally protected from contact with endogenous nucleic acids. However, when in complex with autoantibodies they can, via autoantibody-dependent FcγR-mediated uptake or surface Ig-receptors, get access into endosomes of pDCs and B cells.¹⁴⁻¹⁶ Recognition of ssRNA by TLR7 leads to recruitment of the adaptor molecule myeloid differentiation factor 88 (MyD88), and initiation of a signalling cascade activating the transcription factors interferon regulatory factor (IRF) 7 and NF-κB, finally resulting in gene expression of type I interferons (IFN), IL-6, TNF and other proinflammatory cytokines.¹⁷⁻¹⁹

Type I IFN is an important mediator of the first line of antiviral defence. The pDCs are specialized to rapidly produce large amounts of type I IFN, especially IFN-α, in response to a broad range of viruses and some bacteria.^{19,20} Type I IFN also has powerful immunomodulatory effects that can be deleterious and increase the risk for autoimmune disease in genetically predisposed individuals.²¹⁻²⁴ For example, in systemic lupus erythematosus (SLE) a continuous type I IFN production by pDCs is triggered by an endogenous stimulator consisting of nucleic acid containing immune complexes (IC), for example autoantibodies and nucleic acid binding proteins.²⁴⁻²⁷ In addition, B cells can enhance the IFN-α production by pDCs when stimulated by immune complexes consisting of small nuclear ribonucleoproteins and IgG isolated from SLE patients.⁴ We demonstrated earlier that blocking of the platelet endothelial cell adhesion molecule (PECAM-1/CD31)²⁸ by monoclonal antibodies diminished the stimulatory effect of B cells on the RNA-IC stimulated IFN-α production. However, whether CD31 is involved in the regulation of other proinflammatory cytokines had not been clarified.

Although many synthetic TLR7 agonists have been investigated for their ability to activate immune functions in pDCs and B cells,^{10,29-31} to our knowledge no studies have compared the stimulatory effects of synthetic TLR7 agonists and RNA-containing immune complexes, which mimic endogenous TLR7 activation. Since pDCs and B cells play central roles in several autoimmune diseases and are potential treatment targets, it is important to clarify how the different types of TLR7 agonists affect these cells. In the present study, we stimulated pDCs and B cells, with RNA-IC or a synthetic 8-oxoadenine derivative TLR7 agonist DSR-6434, to compare the pattern

of cytokine production and gene expression in pDCs of 594 immune system-related genes.

2 | MATERIALS AND METHODS

2.1 | Cell isolation and culture conditions

Blood cells were isolated from healthy blood donor buffy coats (Department of Transfusion Medicine, Uppsala University Hospital). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare) density-gradient centrifugation. Due to their inhibitory effect on IFN-α synthesis, CD14-positive monocytes were depleted from PBMC by magnetic bead separation (CD14 MicroBeads (Miltenyi Biotec)).³² pDCs and B cells were isolated from PBMCs using negative selection (pDC Isolation kit II and B cell Isolation kit II; Miltenyi Biotec). Purity of isolated B cells and pDCs was determined by flow cytometry after staining with anti-CD19 (clone HIB19, BD Biosciences) and anti-BDCA2 (Miltenyi Biotec) monoclonal antibodies (mAbs) and was found to be at least 95%. Cells were cultured as previously described⁴ with 0.25×10^4 pDCs alone, or together with 1×10^4 B cells, in volumes of 0.1 mL per well in 96-well plates (Nunc) for 20 hours at 37°C with 5% CO₂. The cells were incubated in the presence of IFN-α2b (500 U/mL; IntronA, MSD) and/or monoclonal antibody to CD31 (PECAM-1), as indicated. The study was approved by the Regional Ethics Committee, and written informed consent was obtained from all blood donors.

2.2 | Cell stimulation

The small molecule TLR7 agonist 6-Amino-2-(butylamino)-9-[[6-[2-(dimethylamino)ethoxy]-3-pyridinyl]methyl]-7,9-dihydro-8H-purin-8-one (DSR-6434, Tocris Bioscience) [EC₅₀ = 7.2 nmol/L]¹² was titrated to achieve optimal induction of IFN-α and was used at a final concentration of 75 nmol/L in subsequent experiments.

U1 snRNP particles were purified from HeLa cells as previously described,³³ and SLE-IgG was isolated from two individual SLE patients' serum containing autoantibodies to SmB, SmD, RNP-A, RNP-C, ribosomal P antigen, histone and dsDNA by protein G chromatography. The U1 snRNP particles and SLE-IgG were used in cell cultures at final concentrations of 2.5 μg/mL and 1 mg/mL, respectively.⁴ To exclude that differences in cytokine production triggered by DSR-6434 and RNA-IC were due to increased cell death, the cell viability was analysed by flow cytometry and showed no difference between the two stimuli. The viability of the pDCs was >80% after overnight incubation independent of stimuli (results not shown).

2.3 | Immunoassays

IFN- α was analysed in culture supernatants by a dissociation-enhanced lanthanide fluoroimmunoassay (DELFI) as previously described.^{34,35} The DELFIA assay does not detect the IFN- α subtype 2b which was used for priming. Cytokines IL-6, IL-8, IL-10 and TNF- α were analysed using the cytometric bead array (CBA) Human Soluble Protein Flex Set system (BD Biosciences) or ELISA immunoassays (BioLegend). The CBA samples were analysed by a FACSCantoII flow cytometer and by FCAP 3.0 software (BD Biosciences).

2.4 | Gene expression profiling

Plasmacytoid dendritic cells from six blood donors were stimulated with DSR-6434, RNA-IC or culture medium (mock), for 6 hours in the presence of IFN- α 2b (IntronA, MSD). Gene expression profiles were analysed by the Human Immunology V2 expression array kit containing probes for 594 immune system-related genes (NanoString Technologies) (Table S1), as described previously.²⁷ Briefly, cell lysates (10 000 cells/5 μ L) were hybridized at 65°C for 18–20 hours, post-hybridized immediately on the nCounter Prep Station, and the signals were then quantified with the nCounter Digital Analyzer (NanoString nCounter platform). The background level was defined as the counts generated by the negative control probes (geometric mean + 3 standard deviations). The raw counts were normalized using internal controls and housekeeping genes (nSolver v3.0 software (NanoString)).

2.5 | Statistics

Statistical analyses were performed using GraphPad Prism Software 7.0. Differences between groups were analysed by Wilcoxon signed-rank test. *P* values $\leq .05$ were considered statistically significant. Differentially expressed genes were defined as absolute log₂ fold change (FC) >2 and Benjamini & Yekutieli adjusted *P* value <.05 (nSolver v3.0).

3 | RESULTS

3.1 | The small molecule TLR7 agonist DSR-6434 induces cytokine production in peripheral blood leucocytes

To investigate whether the small molecule TLR7 agonist DSR-6434 could trigger cytokine production in leucocytes, we stimulated PBMC and CD14-depleted PBMC from

healthy individuals with different concentrations of DSR-6434. The levels of IFN- α , IL-6, IL-8, IL-10 and TNF in the cell cultures were analysed with immunoassays after 20 hours.

Stimulation with DSR-6434 resulted in low or no IFN- α production by both CD14-depleted PBMC (Figure 1A, left panel) and PBMC (results not shown). Next, we asked whether the weak IFN- α production could be enhanced by exogenous IFN- α ('priming'). We found that the IFN- α levels in the cell cultures with monocyte-depleted PBMC were increased 5–6 times with IFN- α 2b priming (Figure 1A, right panel).

We also asked if DSR-6434 could stimulate synthesis of other proinflammatory cytokines. We found that PBMC produced high levels of IL-6 and IL-8 (mean: $\approx 3 \times 10^4$ and 7×10^4 pg/ml, respectively), and moderate levels of TNF (Figure 1B–D). The IL-6 and IL-8 production was lower in the cell cultures lacking monocytes (*P* < .005) (Figure 1E–F), whereas the presence of monocytes did not affect the TNF levels (Figure 1G). In addition, priming with IFN- α 2b reduced the IL-8 production by PBMC (*P* < .0001), whereas TNF production showed a trend to be increased (Figure 1C–D). No IL-10 was detected in the cell cultures (results not shown).

Thus, the TLR7 agonist DSR-6434 triggers high levels of IL-6 and IL-8 in PBMC and monocyte-depleted PBMC, whereas moderate levels of IFN- α by monocyte-depleted PBMC were observed after IFN- α 2b priming.

3.2 | The IFN- α response by plasmacytoid dendritic cells differs between the TLR7 agonists DSR-6434 and RNA-containing immune complexes

Because depletion of monocytes from PBMC and priming with IFN- α 2b increased the DSR-6434-induced IFN- α production, we asked whether pDCs were activated in a similar manner as previously shown for another TLR7 agonist, namely RNA-IC.^{4,32,36} First, we verified that the DSR-6434 concentration titrated in monocyte-depleted PBMC was optimal for IFN- α production by pDCs (Figure S1). Subsequently, pDCs were stimulated with DSR-6434 or RNA-IC in the presence or absence of IFN- α 2b, and the produced IFN- α was quantified in the cell cultures as described earlier.

The pDCs stimulated with RNA-IC produced approximately 20 times higher amounts of IFN- α compared to DSR-6434 as stimulus (median: 2835 vs 131 U/mL, *P* = .001) (Figure 2, left panel). Priming with IFN- α 2b increased the IFN- α production by DSR-6434 stimulated pDCs by approximately threefold (*P* = .0068), whereas the priming had no significant effect on the RNA-IC-stimulated pDCs (Figure 2, left panel).

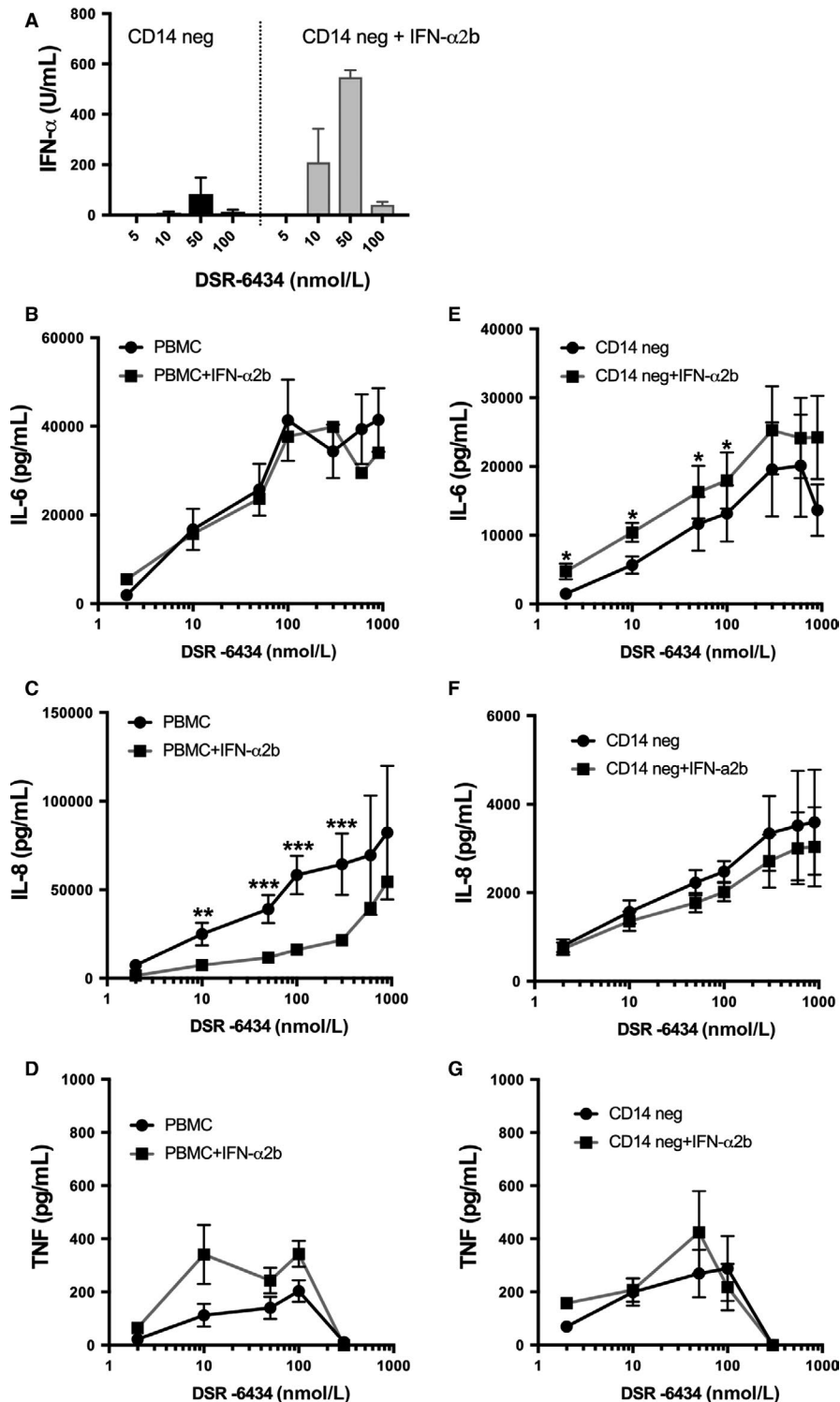


FIGURE 1 Titration of a small molecule TLR7 agonist DSR-6434 and the effect of IFN- α 2b priming on IFN- α , IL-6, IL-8 and TNF production by PBMC and monocyte-depleted PBMC. Peripheral mononuclear cells (PBMC) and CD14-depleted PBMC were incubated with different concentrations of DSR-6434 in the absence or presence of interferon (IFN)- α 2b. (A) IFN- α production by CD14-depleted PBMC, (B, E) interleukin (IL)-6, (C, F) IL-8 and (D, G) TNF production in PBMC and CD14-depleted PBMC. The IFN- α levels were determined at 20h by a DELFIA immunoassay, not detecting the IFN- α 2b subtype, whereas the other cytokines were determined by ELISA immunoassays. Mean values \pm SEM based on 5-8 individual donors from at least four independent experiments are shown. * $P < .05$, ** $P < .01$, *** $P < .001$

B cells are known to potentiate the RNA-IC-induced IFN- α production by pDCs.^{4,27} We therefore asked whether B cells have a similar effect when using DSR-6434 as stimulus. We found that presence of B cells did not regulate the IFN- α production by pDCs, irrespective of IFN- α 2b priming (Figure 2, right panel).

Ligation of the platelet endothelial cell adhesion molecule (PECAM-1/CD31) has been shown to downregulate the

RNA-IC-stimulated IFN- α response.⁴ Therefore, we further asked if CD31 was involved in regulation of the DSR-6434-stimulated IFN- α response. However, we did not find any significant effect of anti-CD31 mAbs on the IFN- α production by pDCs in the presence or absence of IFN- α 2b priming (Figure S2). Furthermore, no detectable levels of IFN- α were produced by B cells, or in mock cultures with medium only (results not shown).

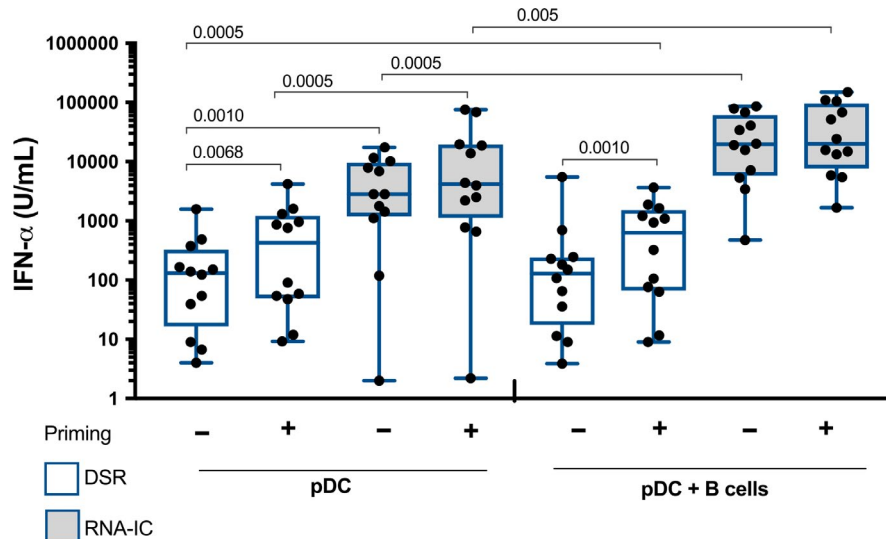


FIGURE 2 IFN- α production by plasmacytoid dendritic cells stimulated with two different TLR7 agonists. Interferon (IFN)- α production by plasmacytoid dendritic cells (pDCs) cultured alone (left panel) or in cocultures with B cells (right panel) isolated from healthy donors was stimulated with two different TLR7 agonists; A small molecule (DSR-6434) or RNA-containing immune complexes (RNA-IC). The cells were cultivated without or with IFN- α 2b (priming). The IFN- α levels were measured in the culture supernatants after 20 h by a DELFIA immunoassay. The box plots show individual values (dots), median (horizontal bars) with interquartile range and minimal and maximal value

Thus, DSR-6434 was a less effective activator of IFN- α synthesis in pDCs than RNA-IC. However, the response was enhanced by exogenous IFN- α 2b priming, but not by B cells, indicating some differences in the regulation of the IFN- α induction by the two TLR7 agonists.

3.3 | IL-6, IL-8 and TNF production stimulated by the TLR7 agonists DSR-6434 or RNA-containing immune complexes

Next, we compared the stimulatory capacity of the two TLR7 agonists, that is the small molecule DSR-6434 and RNA-IC, on IL-6, IL-8 and TNF production by purified pDCs, B cells and in pDC/B cell cocultures. In addition, we examined the role of IFN- α 2b priming.

DSR-6434 induced high production of IL-6, particularly by B cells (mean 1314 pg/mL), whereas RNA-IC only induced IL-6 in the pDC/B cell cocultures (Figure 3A). The DSR-6434-induced IL-6 production was enhanced with IFN- α 2b priming, both in B cells ($P = .002$) and pDCs ($P = .0005$), although the effect was small in pDCs. Coculturing of B cells and pDCs had a stimulatory effect on IL-6 production ($P = .020$), while no additional increase was obtained with IFN- α 2b priming.

Both DSR-6434 and RNA-IC induced IL-8 production by B cells and pDCs (Figure 3B). The IL-8 levels were not significantly different in the B cell cultures stimulated with either TLR7 agonist, whereas DSR-6434 triggered significantly higher IL-8 production by pDCs than RNA-IC (730 vs 100 pg/mL, $P = .0001$). Interestingly, priming of B cells

with IFN- α 2b reduced the IL-8 production ($P < .031$), whereas no such effect was seen in pDCs. Furthermore, there was no additional increase of IL-8 levels in pDC/B cell cocultures.

Hence, DSR-6434 induced higher production of IL-8 in pDCs compared to RNA-IC, whereas almost equal IL-8 levels were synthesized by B cells with either TLR7 agonist.

B cells produced low or undetectable levels of TNF when stimulated with DSR-6434 or RNA-IC, respectively (Figure 3C). In comparison with RNA-IC, DSR-6434 induced higher TNF production in pDCs (570 pg/mL vs 143 pg/mL, $P = .0015$), which was increased twofold by IFN- α 2b priming ($P < .001$). The RNA-IC-induced TNF response was significantly higher in the pDC/B cell cocultures compared to pDCs cultured alone ($P = .0020$), whereas no such increase was observed in the DSR-6434-stimulated cocultures.

In addition, we asked whether ligation of the CD31 surface receptor could regulate IL-6, IL-8 and TNF production. We found that anti-CD31 mAb did not alter the DSR-6434-stimulated IL-6, IL-8 and TNF production (data not shown). In contrast, when using RNA-IC as stimulator, anti-CD31 mAb ligation reduced the levels of all three cytokines, especially in the pDC/B cell cocultures (Figure S3).

In summary, the small molecule TLR7 agonist DSR-6434 effectively triggered IL-6 and IL-8 production by B cells and induced much stronger IL-8 and TNF synthesis in pDCs than RNA-IC. Priming with IFN- α 2b effectively increased the DSR-6434-induced IL-6 and TNF production by B cells and pDCs, respectively.

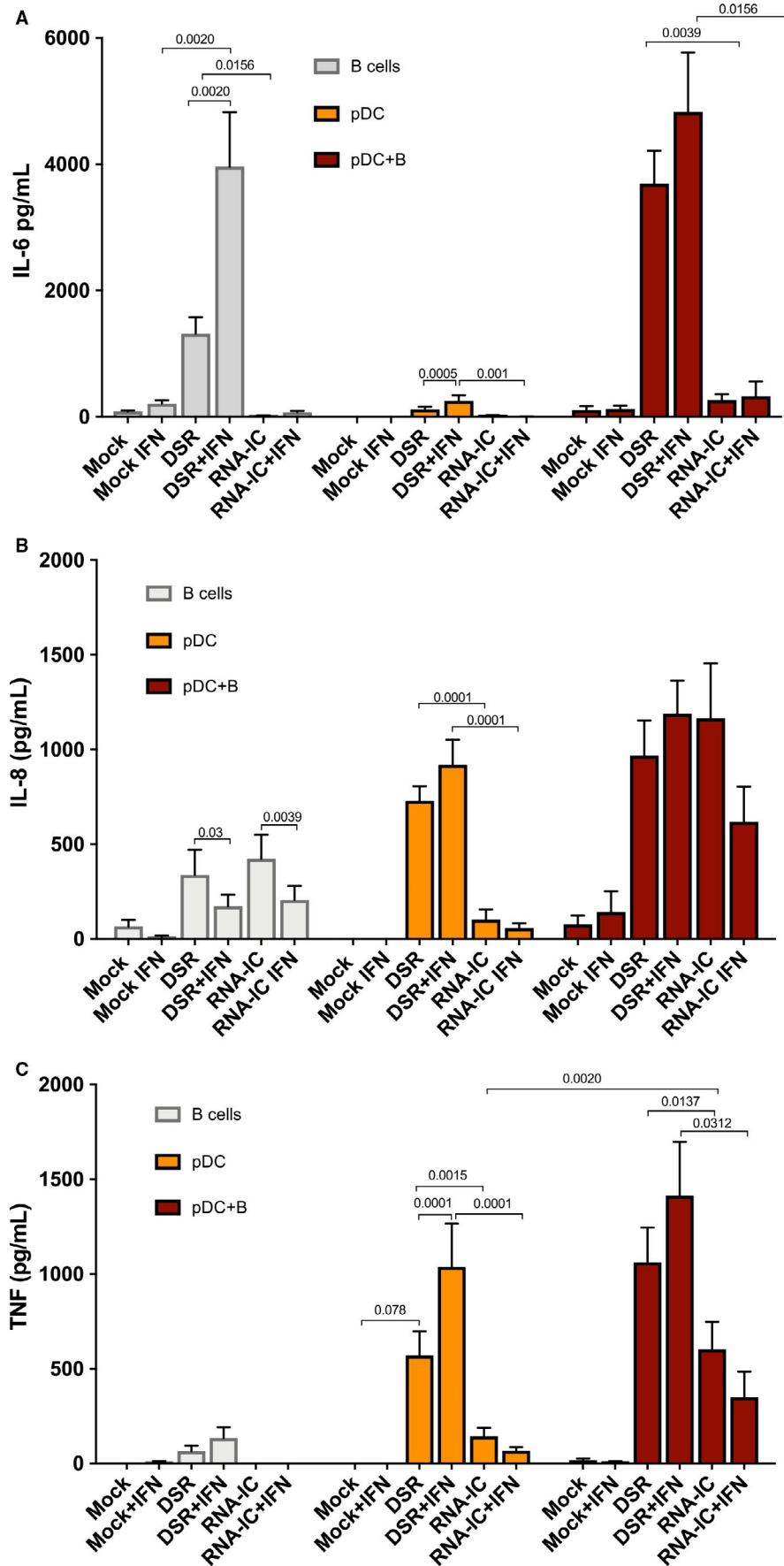


FIGURE 3 Regulation of IL-6, IL-8 and TNF production by plasmacytoid dendritic cells and B cells stimulated with two different TLR7 agonists. (A) Interleukin (IL)-6, (B) IL-8 and (C) tumour necrosis factor (TNF) production by healthy donors' plasmacytoid dendritic cells (pDCs) or B cells cultured alone or in cocultures and stimulated with a small molecule DSR-6434 or RNA-containing immune complexes (RNA-IC). The cells were cultivated in the presence or absence of IFN- α 2b. The cytokine levels were measured in the culture supernatants after 20 h by immunoassays. The bars show mean values and SEM based on at least 6 donors in 4 independent experiments

3.4 | Gene expression profiling of pDCs stimulated with two different TLR7 agonists

To assess the gene expression profile of pDCs upon stimulation with the two structurally different TLR7 ligands, the cells were stimulated with DSR-6434 or RNA-IC for 6 hours in the presence of IFN- α 2b priming and analysed by a NanoString expression array for 594 immune system-related genes. When comparing pDCs stimulated with DSR-6434 and RNA-IC, 12 differentially expressed genes (DEGs) were identified (adjusted P value $< .05$ and absolute \log_2 fold change > 2 , Figure 4 and Table S1). The DSR-6434 induced significantly higher mRNA expression of the costimulatory molecules *CD80* and *CD86*, basic leucine zipper transcription factor (*BATF*), *CCL19*, *STAT5A* and *EBI3* (IL27 subunit beta). RNA-IC on the other hand triggered significantly higher transcript levels of *IL-18R1*, *NADPH oxidase 2 β -chain (CYBB)*, *TGFBR2* and *CXCR4*. Notably, *IL-6* and *IL-8*, which were present at higher protein levels in DSR-6434-stimulated cell cultures (see above), had a tendency of higher expression in the DSR-6434-stimulated pDCs already at the time of RNA sampling (6 hours), but did not reach the significance level.

Comparison of DSR-6434 and mock-stimulated pDCs revealed 56 differentially expressed genes, including 39

upregulated and 17 downregulated transcripts (Figure 4A and Table S1). When comparing RNA-IC and mock stimulated pDCs, 37 DEGs were identified, of which 33 were upregulated and 4 downregulated (Figure 4B and Table S1).

Twenty-four DEGs were upregulated by both TLR7 agonists in comparison with mock stimulation, for example the programmed cell death ligand 1 (*PD-L1/CD274*), *TNFSF10/TRAIL* and lysosomal-associated membrane protein (*LAMP*) 3 as well as signalling molecules *MYD88*, *STAT1* and *STAT2*. Expression of several chemokines (*CCL3*, *CCL4*, *CCR7*, *CXCL9*, *CXCL10* and *CXCL11*) and type I IFN regulated genes was enhanced, as reported earlier for RNA-IC-stimulated pDCs.³⁶ Consistent with the high protein levels of IL-8 and IL-6 measured in 20 hours cell cultures, *NF- κ B* expression was increased by DSR-6434 in comparison with mock stimulated pDCs. Interestingly, *PECAM-1* and *TLR9* were among those genes that were downregulated only in DSR-6434-treated pDCs, whereas expression of *TLR7* and the *interferon regulatory factor (IRF)7* was upregulated by RNA-IC but not by DSR-6434.

In summary, the gene expression analysis of stimulated pDCs showed both unique and shared DEGs for the two TLR7 agonists. This indicates overlapping, but not identical activation pathways via TLR7 for RNA-IC and DSR-6434.

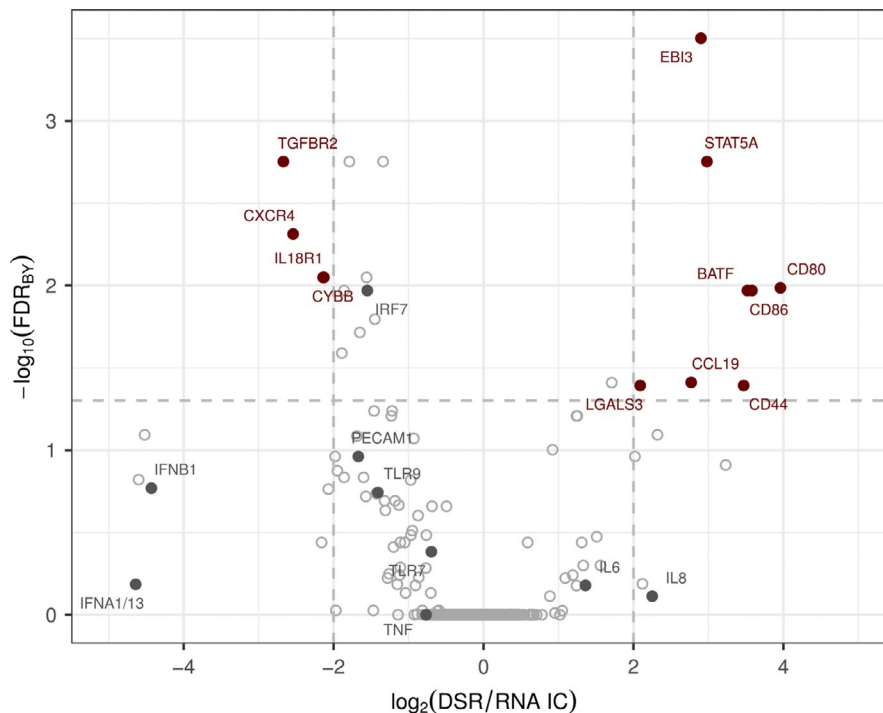


FIGURE 4 Differentially expressed genes in plasmacytoid dendritic cells stimulated with two different TLR7 agonists. Gene expression profile of plasmacytoid dendritic cells (pDCs) isolated from peripheral blood of six healthy individuals and stimulated with a small molecule DSR-6434 or RNA-containing immune complexes (RNA-IC) in the presence of IFN- α 2b priming. Gene expression for 594 immune system-related genes was analysed by a NanoString expression array at 6h. The volcano plot shows gene expression of DSR-6434-stimulated pDCs (right) relative to RNA-IC-stimulated pDCs (left). Significantly differentially expressed genes are highlighted in red, and additional genes of interest are highlighted in grey

Taken together, our data showed that DSR-6434 was a more potent inducer of TNF and IL-8 production by pDCs than RNA-IC, whereas the latter more effectively stimulated pDCs to produce high amounts of type I IFNs. We also showed that IFN- α 2b priming enhanced the weak IFN- α production stimulated by DSR-6434, whereas B cells or ligation of surface CD31 did not have any effect on cytokine production.

4 | DISCUSSION

The main finding in the present study was the observed difference in the activation of proinflammatory pathways between the synthetic TLR7 agonist DSR-6434 and RNA-IC, the latter considered central in the pathogenesis of several systemic autoimmune diseases. An important observation was that the RNA-IC induced almost 20 times higher amounts of IFN- α by pDCs compared to DSR-6434. However, the weak IFN- α response stimulated by DSR-6434 was effectively increased in the presence of IFN- α 2b priming, whereas the latter did not increase the IFN- α production stimulated by RNA-IC, as previously described for the combination of IFN- α 2b and GM-CSF or IL-3.³² In fact, DSR-6434 effectively induced production of IL-6 by B cells, and both IL-8 and TNF by pDCs. This highlights that the stimulatory capacity of even a weak IFN- α inducer, such as DSR-6434, can be potentiated in the presence of endogenous type I IFN and proinflammatory cytokines, which might not be desirable in individuals prone to autoimmunity.

It has previously been shown that IFN- α and IFN- β have an inhibitory effect on IL-8 production by PBMC and fibroblasts.^{37,38} We confirmed this finding in DSR-6434-stimulated PBMC primed with IFN- α 2b. Furthermore, we demonstrated that IL-8 production was reduced by IFN- α 2b priming in B cells, but not in pDCs. Interleukin-8 is produced by various cell types exposed to microorganisms and inflammatory stimuli and is the principal chemoattractant for neutrophils.³⁹ Activated neutrophils have been shown to form neutrophil extracellular traps (NETs) and effectively induce inflammation and type I IFN synthesis in pDCs.⁴⁰ Notably, RNA-IC increased the expression of *CYBB*, which is a component of NADPH oxidase, and it has been reported that IL-8 potentiates the oxidative burst contributing to rapid production of reactive oxygen species. The mechanism for downregulation of IL-8 has not been clarified, but a possible resistance of pDCs to downregulate their own IL-8 production in an auto-crine manner has been suggested, which could add on to the inflammatory cascade driven by activated pDCs. It has been shown that TNF and IL-1 can induce IL-8 production,³⁷ but in our study only low levels of TNF was produced by the B cells. This indicates that TNF was not the major stimulator of IL-8 in our study.

In contrast to RNA-IC, the DSR-6434-induced IFN- α synthesis by pDCs was not enhanced by cocultivation with B cells, nor reduced by monoclonal antibodies to PECAM-1/CD31.⁴ This demonstrates a different regulatory pattern of IFN- α synthesis between the two TLR7 agonists. This is in line with our finding that CD31, which normally is expressed by both pDCs and B cells, was downregulated in pDCs stimulated with DSR-6434.

The observed differences in the IFN- α inducing capacity between the two TLR7 agonists could be mediated by increased TLR7 expression in pDCs after the RNA-IC stimulation. This could be due to high levels of IFN- β induced by RNA-IC, which is reported to upregulate the TLR7 expression in pDCs.⁴¹ One could further speculate that DSR-6434 and RNA-IC have different capacities to localize to and remain in the endo-lysosomal compartments and that RNA-IC can sustain a robust TLR7 expression in pDCs available for type I IFN induction. A previous study showed that repeated administration of DSR-6434 into mice downregulated TLR7 expression, independently of signalling via the type I IFN receptor.⁴² Although we did not investigate the cellular localization of the RNA-IC and DSR-6434, it was clear that both TLR7 agonists were acting via endosomes. This was because hydroxychloroquine, which inhibits acidification of the endosomal compartments, blocked IFN- α production by pDCs stimulated with DSR-6434 (results not shown) similarly as previously demonstrated for RNA-IC-stimulated pDCs.^{16,36}

We also found that TLR9 gene expression was higher in RNA-IC-stimulated pDCs and was in fact reduced by DSR-6434 treatment when compared with mock stimulated pDCs. Such variation could have impact on the balance between TLR7 and TLR9 signalling, a balance that has been shown to be important in the regulation of the inflammatory response.⁶ In addition, we found that RNA-IC induced particularly high gene expression of IFN- β , whereas both TLR7 agonists effectively triggered gene expression of several chemokines and chemokine receptors. Particularly, CXCL10 and CXCL11 were highly upregulated. Both bind to CXCR3 and primarily play a chemotactic role for lymphocytes and regulate differentiation towards a Th1 profile.⁴³ The DSR-6434 triggered higher expression of NF- κ B in pDCs which is consistent with the higher levels of IL-8 and TNF, whereas RNA-IC induced higher expression of IRF7 and a more potent type I IFN response.

We conclude that the TLR7 ligands, a small molecule DSR-6434 and RNA-containing ICs, activate pDCs and B cells to produce several inflammatory cytokines, but the expression and regulation of their synthesis differ. Thus, a careful selection of relevant immune cell activators or inhibitors is necessary when identifying possible targets for modulation of the immune response in patients with autoimmune diseases or a compromised immune system.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTION

OB, CA, MLE performed the experiments and collected data. PP, CA, OB, LR and MLE analysed and interpreted the data. MLE, PP and LR wrote the manuscript. All authors read and approved the final version of the manuscript. MLE supervised the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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