Interferon-alpha Mediates Suppression of C-Reactive Protein Explanation for Muted C-Reactive Protein Response in Lupus Flares?

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Interferon alpha mediates suppression of C-reactive protein (CRP)
-Explanation for muted CRP response in lupus flares?

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**Objective.** C-reactive protein (CRP) is synthesized by hepatocytes in response to interleukin-6 (IL-6) during inflammation. Despite raised IL-6 and extensive systemic inflammation, serum CRP levels remain low during most viral infections and disease flares of systemic lupus erythematosus (SLE). Since both viral infections and SLE are characterized by high levels of interferon alpha (IFNα) we asked whether this cytokine can inhibit CRP induction.

**Methods.** The interference of all 12 IFNα subtypes with CRP-promoter activity, induced by IL-6 and IL-1β was studied in a CRP-promoter and luciferase-reporter transfected human hepatoma cell-line, HepG2. CRP secretion by primary human hepatocytes was analyzed by enzyme-linked immunosorbent assay.

**Results.** The CRP-promoter activity was inhibited by all single IFNα subtypes, as well as two different mixtures of biological relevant IFNα subtypes. The most prominent effect was seen using a leukocyte-produced mixture of IFNα (56% inhibition at 1000 IU/mL). The inhibitory effect of IFNα was confirmed in primary human hepatocytes. CRP-promoter inhibition was dose-dependent and mediated via the type I IFN receptor. Transferrin production and HepG2 proliferation/viability was not affected by IFNα.

**Conclusion.** Our study demonstrates that IFNα is an inhibitor of CRP-promoter activity and CRP secretion. This finding concords with previous observations of up-regulated IFNα and muted CRP response in SLE flares. Given the fundamental role of both IFNα and CRP in the immune response, our results are of importance for the understanding of the pathogenesis of SLE, and may also contribute to the differences in CRP response between viral and bacterial infections.
The pentraxin C-reactive protein (CRP) is an acute phase protein, produced by hepatocytes in response to inflammation and/or tissue damage (1). Its rapid and profound increase makes it a widely used marker of acute inflammation and to evaluate the inflammatory state in chronic diseases like rheumatoid arthritis (RA). Serum CRP levels are also used to distinguish bacterial from viral infections, since bacterial infections generally yield a more pronounced CRP elevation.

CRP synthesis is mainly induced by interleukin (IL)-6, which mediates transcription through the signal transducer and activator of transcription 3 (STAT3) and the CCAAT box/enhancer binding protein C/EBPβ and δ (1). An additive effect of IL-1β is seen, primarily in hepatoma cell lines, and is thought to involve the binding of nuclear factor-kappaB to the CRP-promoter (2).

CRP is a potential protector in systemic inflammatory diseases like systemic lupus erythematosus (SLE). For instance, CRP may act as an opsonin by decorating bacterial surfaces, dying cells, and apoptotic debris by affinity for phosphoryl choline (PC) and nuclear constituents, and by its interaction with complement and Fc-gamma receptors (FcγRs) I and IIa/IIb (1). Thereby it protects the adaptive immune system from exposure to dying cells, induction of autoantibodies and eventually systemic autoimmune diseases (1, 3). CRP also interacts with nuclear antigens that are targets for antinuclear antibodies (ANA) in SLE (1). Single nucleotide polymorphisms of the CRP gene, have been found to associate with low baseline levels of CRP, ANA production and increased susceptibility to SLE (4). Furthermore, a delayed disease onset, reversed nephritis, and prolonged survival in murine lupus models treated with CRP (5), indicates a preventive and disease-modifying role.
Despite extensive systemic inflammation and raised IL-6 levels, serum CRP typically remains low (~15 mg/L) in disease flares, but is substantially raised during bacterial infections in SLE patients (6, 7). Although anti-CRP autoantibodies are common in SLE, they do not target native circulating CRP (8) and the CRP-consumption rate in SLE patients does not differ from that of healthy individuals (9). In addition to low CRP, SLE patients typically have raised blood levels of IFNα during disease flares (10). Since IFNα characterizes both viral infections and SLE flares, two conditions where CRP is relatively low, we asked whether IFNα could act as an inhibitor of CRP synthesis. In the present study we therefore examined the effects of different IFNα subtypes as well as virus and immune complex-induced IFNα mixtures on the CRP-promoter activity and secretion.
Materials and Methods

HepG2 reporter gene assay and primary hepatocytes

CRP-gene regulation was studied a human hepatoma cell line (HepG2), stably transfected with 1-kb CRP-promoter and a luciferase-reporter gene construct (HepG2-ABEK14 cells) kindly provided by Dr. Jan Torzewski (Ulm, Germany (11)). HepG2-ABEK14 were cultured in RPMI 1640 medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine (Invitrogen, San Diego, CA) and 10% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO) and were routinely screened for mycoplasma (Mycoalert, Lonza, Rockland, ME). Cells at a confluence of 50% were stimulated in triplicates as indicated for 24 hours. IntronA (IFNα2b) was purchased from Schering-Plough (Kenilworth, NJ) and IL-1β, IL-6 and mouse IgG2A isotype control from R&D Systems (Abingdon, UK). Other recombinant IFNα subtypes and neutralizing mouse anti-human IFNAR chain 2 (clone MMHAR-2) were from PBL InterferonSource (Piscataway, NJ). Endotoxin levels were <1EU/µg in antibody/protein preparations. In receptor blocking experiments, the cells were pre-incubated with antibodies (50 µg/mL) two hours prior to cytokine addition. Stimulated cells were lysed with Glo lysis buffer (Promega, Madison, WI), then mixed with Bright glo assay reagent (Promega), and luminescence was recorded. Ratios for stimulated cells versus untreated are shown as fold increase in CRP-promoter activity.

Primary human hepatocytes (Clonetics Ready Heps), freshly isolated from healthy parts of resected liver tissue and cultured in collagen-coated 24-well plates were obtained from Lonza. The cells were maintained in hepatocyte culture medium (Lonza) and were stimulated in duplicates as indicated for 24 hours.
Peripheral blood mononuclear cells (PBMC) from healthy donor buffy coats were isolated by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density-gradient centrifugation. Plasmacytoid dendritic cells (pDC) were purified (>95% purity by BDCA-2 staining) from PBMC by negative depletion using the pDC Isolation kit and MACS LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured in Macrophage-SFM medium (Invitrogen) supplemented with HEPES (20mM), penicillin (60µg/mL) and streptomycin (100µg/mL).

U1 small nuclear ribonucleoproteins (snRNP) particles were purified (>90% purity) from HeLa cells as described by Bach et al (12). Serum from an SLE patient with antibodies to Sm, RNP, ribosomal P antigen, histones and dsDNA was used to prepare IgG by protein G chromatography. The U1 snRNP particles were used at a concentration of 2.5µg/mL in the pDC cultures together with 1 mg/mL of IgG. Supernatants were collected after 20 hours of incubation. PBMC were stimulated with Sendai virus and the cell culture supernatant was purified as described (13). The IFNα concentrations of purified IFNα and in pDC supernatants were determined by dissociation-enhanced lanthanide-fluoroimmunoassay (DELFIA) (14).

Cell culture supernatants for protein analysis by enzyme-linked immunosorbent assays (ELISA) were centrifuged and stored at -70ºC. Commercial ELISAs were used to analyze CRP (R&D Systems), transferrin (Universal Biologicals, Cambridge, UK) and serum
amyloid A (SAA) (BioSupply UK, Bradford). Stimulation of HepG2-ABEK14 for SAA secretion was performed in serum free medium.

Cell proliferation

The relative number of viable cells was determined by a tetrazolium-based cell proliferation assay (Celltiter 96 aqueous one solution cell proliferation assay, Promega) according to the manufacturers’ instructions.

Statistics

Statistical significance was evaluated using paired Student’s t-test or One-way ANOVA repeated measurements with Dunnett’s post test. A p-value of <0.05 was considered significant.
Results

*IFNα inhibits CRP-promoter activity in HepG2-ABEK14*

The CRP promoter in HepG2-ABEK14 was activated by combined stimulation with IL-1β and IL-6, but responded weakly to IL-1β or IL-6 alone (Fig 1A). The IL-1β/IL-6 co-mediated CRP-promoter activity reached a plateau (17-fold) at IL-1β/IL-6 concentrations of 6ng/mL and this concentration was henceforth selected for CRP-promoter induction.

All 12 IFNα subtypes showed a concentration-dependent inhibitory effect on the CRP-promoter activity (Fig 1B), ranging from 24.5% for IFNα17 to 47.4% for IFNα10 at 1000 IU/mL. To mimic the naturally occurring spectrum of IFNα which is produced during viral infections or in SLE flares, two different mixtures of IFNα were used. Purified leukocyte IFNα (PLIFN) from Sendai-virus stimulated PBMCs, and supernatants from snRNP IC-stimulated pDCs (pDC-sup) suppressed the CRP-promoter activity by 56.4% and 49.1% respectively (Fig 1C). IFNα2b (IntronA) inhibited the promoter activity by 40.3% (Fig 1C).

*Time course*

Time course studies revealed that the CRP-promoter is activated within 2 hours after IL-1β/IL-6 stimulation both in the absence and presence of IFNα2b (Fig 1D). In the IFNα2b treated cells an inhibition of the promoter activity followed about 6 hours after IL-1β/IL-6 stimulation. Pre-incubation with IFNα2b for 6 hours resulted in suppressed promoter activity from the start of IL-1β/IL-6 stimulation.
The effect of IFNα is mediated via the type I IFN receptor

A neutralizing antibody to the type I IFN receptor (IFNAR) was used to assess the role of the receptor in IFNα-dependent inhibition of CRP-promoter activity. Compared to an isotype-matched control, this antibody almost completely restored CRP-promoter activity (Fig 2).

CRP secretion is inhibited by IFNα2b in primary hepatocytes

The IFNα-dependent inhibition of CRP-promoter activity was confirmed by studies of CRP secretion in primary human hepatocytes. IL-1β-induced CRP secretion was inhibited by 49.2% and IL-6 induced secretion was inhibited by 51.5% whereas the inhibition was moderate (21.1%) in IL-1β and IL-6 co-induced CRP release (Fig 3A).

Serum amyloid A, but not transferrin or viability, is affected by IFNα

Although HepG2 cells have lost their ability to produce CRP, they still have the capacity to synthesize the related acute phase protein SAA. Therefore, we investigated whether or not SAA synthesis could be suppressed by IFNα in a similar fashion to CRP. At a concentration of 1000 IU/mL, IFNα2b reduced the SAA release from HepG2-ABEK14 by 38.4% (Fig 3B) similar to the reduction of CRP-promoter activity (40.3%).

To exclude general effects of viability or protein synthesis, we investigated the potential effect of IFNα2b on cell proliferation and secretion of transferrin (a negative acute phase protein) in HepG2-ABEK14. Using the same conditions as for the CRP-promoter studies (i.e. 24 hours stimulation with 1000 IU/mL), we found no detectable suppression of proliferation/viability due to IFNα2b (Fig 3C). Transferrin levels were reduced by IL-1β/IL-6, but remained unaffected by IFNα2b (Fig 3D).
Discussion

The main finding in this *in vitro* study is that all IFNα subtypes, as well as two different mixtures of biological relevant IFNα subtypes, can inhibit IL-1β/IL-6 co-induced CRP-promoter activity. The inhibiting effect of IFNα was confirmed by studies of CRP secretion in primary hepatocytes. IFNα mediated its effect via IFNAR and we observed a dose-dependent reduction of the promoter activity at IFNα concentrations within a range relevant for autoimmune disease states such as SLE (10) and therapeutic administration of IFNα2b (15). Although our conclusions are based on *in vitro* studies, the findings are of interest in relation to SLE pathogenesis, since these patients typically express a dominant pattern of type I IFN-inducible gene expression (‘the IFN signature’), and during disease flare display increased levels of circulating IFNα (10, 16), a weak CRP response and no correlation between CRP and IL-6 levels (6, 7). Consequently, it is likely that IFNα impedes CRP responses in SLE and many viral infections.

Human type I IFNs mainly comprise IFNβ, IFNω and 12 subtypes of IFNα, all interacting with the same receptor, IFNAR. These type I IFNs have a high degree of homology and exhibit comparable biological effects such as immunostimulatory as well as immunoregulatory functions that may participate in the induction and maintenance of autoimmune/inflammatory responses (16). The therapeutic efficacy of IFNα in cancer has been attributed to its anti-proliferative properties. However, such effects do not explain our observed IFNα-mediated reduction in CRP-promoter activity, since neither cell proliferation nor secretion of transferrin was affected.
We found that inhibition of IL-6/IL-1β induced CRP-promoter activation occurred within 6-8 hours after exposure to IFNα, indicating that de novo protein synthesis might be required for inhibition. However, the detailed mechanisms behind IFNα-induced CRP-promoter inhibition have not yet been unraveled but, as discussed in a recent review by Gaitonde et al, several possibilities exist (17). Studies on this matter are being carried out at our laboratory.

CRP possesses powerful anti-inflammatory properties, and several protective functions of CRP have been suggested in relation to SLE. In the murine lupus model MRL/lpr, single CRP-injections of human CRP resulted in reduced levels of anti-DNA autoantibodies, reversal of proteinuria and prolonged survival (5). Long-term protection was dependent on CD25, suggesting that the effect was mediated by regulatory T-cells. CRP-induced complement activation is another potential mode of CRP-regulated inflammation. Complement has the ability to cause severe inflammation if not tightly regulated. However, CRP recruits factor H, an inhibitor of C3-dependent later events, and CRP-induced complement activation therefore results in less formation of membrane attack complex and C3b-induced amplification of the alternative pathway (3).

Considering all these CRP-mediated effects, our finding that IFNα hampers hepatocyte CRP induction may be a clue to pathogenic mechanisms in SLE. In addition, treatment with CRP-reducing strategies, for instance anti-IL-6, could hypothetically have negative consequences with regard to the risk of precipitating SLE. Increased understanding of the IFNα-CRP interplay and homeostasis is therefore important to gain more detailed insights into immunoregulatory mechanisms in systemic immune-mediated diseases like SLE, as well as in malignancies, infections and cardiovascular disease.
To conclude, we present results suggesting that ongoing production of IFNα in SLE patients could be responsible for a muted CRP response in SLE flares. This may have important implications regarding the pathogenesis of SLE and perhaps also other systemic rheumatic diseases characterized by an IFN signature, since emerging data suggest a protective function of CRP in SLE. Further studies of the interaction between the type I IFN system and pentraxins, including CRP, are therefore warranted and may be valuable when new treatment strategies for SLE are developed.

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References


Figure 1. CRP-promoter activity in HepG2-ABEK14. A, Combined stimulation with IL-1β and IL-6 (24 h) strongly induced CRP-promoter activity compared to untreated control cells, whereas IL-1β or IL-6 alone generated a modest increase. B, Inhibition of the IL-1β/IL-6-induced (6ng/mL) CRP-promoter activity by 12 IFNα subtypes (24 h), organized with regard to inhibitory effect. C, Inhibition of the IL-1β/IL-6-induced (6ng/mL) CRP-promoter activity by purified IFNα from virus-stimulated leukocytes (PLIFN), supernatants from plasmacytoid dendritic cells exposed to snRNP immune complexes (pDC-sup) or IntronA (IFNα2b) (24h). D, Time course of the CRP-promoter activation in HepG2-ABEK14 cells after stimulation with IL-1β and IL-6 in the presence or absence of IFNα2b and after pre-incubation with IFNα2b. Data is presented as mean fold increase in CRP-promoter activity ± SEM for three (panel B, C and D) or four (panel A) independent experiments. Asterisks indicate differences between stimulated cells (IFNα 100/1000 IU/mL) and unstimulated cells. *** = p<0.001.
**Figure 2.** The effect of a neutralizing antibody to the type I IFN receptor (IFNAR). HepG2-ABEK14 cells were pre-incubated with 50µg/mL of antibody, and stimulated with IL-1β, IL-6 (6ng/mL) and IFNα2b (100 IU/mL). The mean (± SEM) inhibitory effect of IFNα2b is shown for three separate experiments. ** = p<0.01.
Figure 3. IFNα2b-mediated effects on acute phase protein synthesis and cell proliferation/viability. A, Effects of IFNα2b on CRP secretion by primary human hepatocytes. CRP secretion was induced by 6ng/mL of IL-1β, IL-6 or IL-1β and IL-6 in combination. B, Serum amyloid A (SAA) secretion by HepG2-ABEK14, induced by 6ng/mL of IL-1β and IL-6. C, Cell viability/proliferation in HepG2-ABEK14. D, Secretion of the negative acute phase protein transferrin by HepG2-ABEK14. Data is from two (panel A), one (panel B) or three (panel C and D) independent experiments. Results are mean ± SEM.