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CHAPTER 7

Inhibition of the type I immune responses of human monocytes by IFN-α and IFN-β

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Abstract

Interleukin-12 (IL-12), IL-23 and interferon-γ (IFN-γ) are pivotal cytokines acting in concert with tumor necrosis factor (TNF) and IL-1β to shape type I immune responses against bacterial pathogens. Recently, several groups reported that type I immunity can be inhibited by IFN-α/β. Here we show the extent of the inhibitory effects of IFN-α and IFN-β on the responsiveness of human monocytes to Toll like receptor-ligands and IFN-γ. Both IFN-α and IFN-β strongly reduced the production of IL-12p40, IL-1β and TNF and the IFN-γ induced CD54 and CD64 expression. High IFN-γ concentrations could not counterbalance the inhibitions and IFN-α still inhibited monocytes 24 h after stimulation in vitro as well as in vivo in patients undergoing IFN-α treatment. Next, we explored the mechanism of inhibition. We confirm that IFN-α/β interferes with the IFN-γR1 expression, by studying the kinetics of IFN-γR1 downregulation. However, IFN-γR1 downregulation occurred only after two hours of IFN-α/β stimulation and was transient, which can not explain the IFN-γ unresponsiveness observed directly and late after IFN-α/β stimulation. Additional experiments indeed indicate that other mechanisms are involved. IFN-α may interfere with IFN-γ-elicited phosphorylation of signal transducer and activator of transcription 1 (STAT1). IFN-α may also activate methyltransferases which in turn reduce, at least partly, the TNF and IL-1β production and CD54 expression. IFN-α also induces the protein inhibitor of activated STAT1 (PIAS1). In conclusion, IFN-α and IFN-β strongly inhibit the IFN-γ responsiveness and the production of type I cytokines of monocytes, probably via various mechanisms. Our findings indicate that IFN-α/β play a significant role in the immunopathogenesis of bacterial infections, for example Mycobacterium tuberculosis infection.
1. Introduction

IFN-α and IFN-β as well as IFN-γ display immunomodulatory effects to help the host to combat infections. IFN-α and IFN-β are closely related cytokines, which are important in the defence against viruses. IFN-γ is the main mediator of the type I immune response and is essential in the control of infections with intracellular pathogens, such as *Mycobacteria* and *Salmonellae* [1]. IFN-α and IFN-β also play a role in various bacterial diseases, for example tuberculosis. Patients with active infection with *Mycobacterium tuberculosis* were recently shown to have an expression profile typical of IFN-α-induced immune related genes coinciding with reduced IFN-γ signalling within their blood cells [2, 3]. This is in line with the finding that virulent *Mycobacterium tuberculosis* strains isolated from humans induced IFN-α production in mice, which correlated with decreased type I immunity [4, 5]. Thus, *Mycobacterium tuberculosis* survival in the host may benefit from enhanced IFN-α/β signalling and repressed IFN-γ signalling. In addition, virulent strains of the intracellular pathogens *Bordetella pertussis* and *Francisella tularensis* were also found to inhibit the type I immune responses of human dendritic cells, via induction of IFN-β [6, 7].

IFN-α/β and IFN-γ have both common and distinct effects on human cells [8, 9]. IFN-α/β act inhibitory on immature and stimulatory on mature antigen presenting cells, B- and T-lymphocytes [10]. Therefore IFN-α/β may favour late antibacterial responses [11], although IFN-α/β may actually dampen antibacterial responses in the early phase of infection. For example, IFN-β exposure during naïve T cell stimulation inhibits Th1 cell generation by inhibiting the IL-12 and IL-23 production of cultured human dendritic cells [12]. We recently reported that IFN-α can also reduce the IFN-γ responsiveness of human primary monocytes [13]. Yet, little is known about the precise extent and mechanisms whereby IFN-α/β inhibits type I immune responses. IFN-α/β may very well interfere with the control of infections with intracellular pathogens. The inhibitory effects of IFN-α/β may thus explain why in influenza virus infected mice, virus-induced IFN-α renders the mice highly susceptible to bacterial infections [14], and why mice that lack a functional IFN-α/β receptor are relatively resistant to infections with the intracellular pathogen *Listeria monocytogenes* [15].

Upon IFN-γ stimulation, via the IFN-γR, STAT1 is tyrosine phosphorylated, dimerizes and translocates to the nucleus. STAT1 homodimers can activate transcription of several genes via binding to IFN-γ activated sequences (GAS) in promoters [16]. IFN-α and IFN-β both signal via the IFN-α receptor complex (IFN-αR) resulting in STAT1-STAT2 heterodimers, which associate mostly with interferon regulatory factor 9 (IRF-9) to form interferon-stimulated gene factor 3 (ISGF3) complexes [17]. The transcription factor ISGF3 binds to interferon-stimulated response elements (ISREs), while STAT1-STAT2 heterodimers can also bind to certain GAS sites [9]. IFN-α/β and IFN-γ signalling may thus result in both common and distinct responses. A broad range of genes can be induced by both IFN-γ and IFN-α, albeit
with different efficacy [8]. For example, the transcription factor IRF-1 can be effectively induced by both IFN-α and IFN-γ. On the other hand, IFN-γ upregulates IRF-8 [18], CD54 and CD64 expression and enhances LPS-induced cytokine production of interleukin-1β (IL-1β), IL-12, IL-23 and TNF [13], while IFN-α on the contrary inhibits these IFN-γ effects.

Inhibition of IFN-γ responses by IFN-α/β could potentially be achieved via for instance IFN-γ receptor (IFN-γR) downregulation, prevention of STAT1 homodimer formation or activation of protein arginine methyltransferase 1 (PRMT1). PRMT1 was found to be associated with the IFN-αR1 subunit of the IFN-αR [19], which may indicate that PRMT1 is regulated by IFN-α/β [20]. Activation of PRMTs results in methylation of arginine residues of various proteins, thereby modulating their actions [21]. PRMT1 can, amongst others, methylate the protein inhibitor of activated STAT1 (PIAS1) [22]. Methylated PIAS1 binds to STAT1, thereby negatively influencing the DNA binding capacity of STAT1 homodimers to certain, but not all, GAS sites [23]. In this way PIAS1 selectively inhibits IFN-γ induced transcription of genes.

In this study, we focused first on the extent of the opposing effects of IFN-α and IFN-β on the type I immune responses of human monocytes in vitro. We investigated whether IFN-α and IFN-β display comparable opposing effects, and whether high doses of IFN-γ can overcome these effects. Second, we determined the duration of the inhibitory effects in vitro and in vivo. Third, we explored by which mechanism IFN-α can interfere with IFN-γ functions, by examining the effects of IFN-α on IFN-γR expression and STAT1 phosphorylation. Additionally, we investigated whether PRMT1 and PIAS1 could play a role in the inhibitory effects of IFN-α.

2. Materials and Methods

2.1. Monocyte isolation and culture

PBMCs were isolated from blood of healthy blood bank donors, via ficoll separation. Subsequently monocytes were isolated using CD14 MACS beads (Miltenyi) according to the manufacturer’s protocol. The isolated monocytes contained less than 1.5 % CD3+ cells, as analysed by FACS using PE conjugated antibody against CD3 (BD Biosciences). Cells were cultured in 96 wellsplates in IMDM (Lonza) supplemented with 8% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM glutamax (Life Technologies). Cells were stimulated, as indicated in each experiment, with 1 ng/ml LPS (Sigma), 10 µg/ml of heat killed *Mycobacterium tuberculosis* (MTB) (kind gift from L. Wilson), IFN-α, IFN-β, IFN-γ (PBL) and Arginine Methyltransferase Inhibitor 1 (AMI1, Calbiochem). The IFN-α is a 1:1 mixture of the subtypes 2a and 2b. The IFN-β is of the 2b subtype. 10 Units/ml IFN-α/β equals the concentration of 94 pg/ml IFN-γ.
2.2. Patient materials and immunological screenings
Patients with cutaneous melanoma received IFN-α treatment prior to and after T cell infusions [24]. IFN-α (subtype 2a, 3 million IU Roferon, Roche) was injected subcutaneously each day. PBMCs were isolated from blood and stored in liquid nitrogen until use at two time points: once before the start of the treatment and once 2 to 4 weeks later during IFN-α treatment. Thawed PBMCs were washed three times with culture medium and directly used in further assays using 96 wellsplates (Greiner bio-one). The IFN-γR1 receptor expression and the IFN-γ induced CD54 expression were analyzed by FACS as described in section 2.3, except that the cells were also stained with FITC conjugated CD14 (BD Biosciences), in order to assess the expression on CD14+ monocytes. The LPS responsiveness was tested by stimulating 2*10^5 PBMCs (the standard deviation of cell counting was about 5%), from patients and controls, with or without 1 ng/ml LPS in 200 µl of IMDM supplemented with 8% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM glutamax. After 24h of incubation supernatants were harvested and analyzed for the presence of IL-12p40 by ELISA (Invitrogen). All patients gave written informed consent. The treatment protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center.

2.3. Analysis of receptor expression
1.5*10^5 PBMCs or CD14+ monocytes were incubated with various concentrations of IFN-γ or IFN-α in 200 µl of culture medium. Cells were washed with PBS supplemented with 0.2% bovine serum albumin (BSA) (Roche). To determine CD54 and CD64 expression the cells were stained with PE labelled anti-CD54 and FITC labelled anti-CD64 (clones HA58 and 10.1, BD Biosciences). To determine the IFN-γR1 expression the cells were stained with PE labelled GIR94 (BD Biosciences). Cells were analyzed with a FACSCalibur using CellQuest software (BD Biosciences).

2.4. Measurements of cytokine production
1.5*10^5 CD14+ monocytes were incubated with various concentrations of IFN-γ and/or IFN-α in the presence or absence of 1 ng/ml LPS. After 24h supernatants were collected and analysed for the presence of IL-12p40, IL-1β and TNF by ELISA (Invitrogen) using microlon plates (Greiner bio-one). The detection limit of the ELISAs was 150 pg/ml.

2.5. FACS analysis of STAT1 phosphorylation
2*10^5 CD14+ monocytes were incubated for 5 to 60 minutes with various concentrations of IFN-γ and/or IFN-α in 200 µl of culture medium. In order to detect intracellular phosphorylation of STAT1, cells were fixated with 4% paraformaldehyde (Sigma) and permeabilized with 90% methanol (Merck). Subsequently, the cells were treated with 10% normal goat serum (Sanquin) and stained with Alexafluor-647 labelled mouse-anti-human-
pY701-STAT1 (clone 4a, BD Biosciences). After labelling, the cells were washed twice and
analyzed by FACS using a FACSCalibur and CellQuest software (BD Biosciences).

2.6. Western blot analysis of STAT2 phosphorylation
To detect STAT2 tyrosine phosphorylation, we incubated 2*10^6 CD14+ monocytes with
100 U/ml IFN-α and/or 2.5 ng/ml IFN-γ. After incubation the cells were washed with an
excess of ice-cold PBS. The cells were lysed in a radioimmune-precipitation-assay (RIPA)
buffer supplemented with PMSF, protein inhibitors and sodium-orthovanadate, according
to the supplier’s recommendations (SantaCruz). Equal protein amounts, determined using a
Bradford assay (Thermo Scientific), were run on a 10% polyacrylamide gel (Promega), and
blotted on a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer) using electrophoresis
and blotting apparatuses according to the manufacturer’s protocols (BioRad). Membranes
were blocked for 1 h in PBS supplemented with 5% milk powder (Campina). Subsequently,
the membranes were incubated with mouse-anti-human-pY690-STAT2 (clone 7a, BD
Biosciences) in PBS-2.5% milk powder, washed and incubated with a HRP-conjugated Fab
fragment of a goat-anti-mouse antibody (SantaCruz) in PBS-2.5% milk powder. As a control
the blot was subsequently incubated with a HRP-conjugated antibody against GAPDH (clone
Fl335, SantaCruz). Binding was detected using an enhanced chemiluminescence kit (Thermo
Scientific) and exposure to X-ray film (Fuji).

2.7. Western blot analysis of PRMT1 and PIAS1
The expression of PRMT1 and PIAS1 was analyzed by western blot as described above, with
some slight modifications. Tris-buffered-saline (Roche) instead of PBS was used to incubate
the PVDF membranes after blotting. Specific monoclonal rabbit-anti-human antibodies
against PRMT1 and PIAS1 (clone A33 and clone D33A7, Cell Signalling Technologies) and
a secondary HRP-conjugated donkey-anti-rabbit antibody (sc-2077, SantaCruz) were used to
determine expression. As a control GAPDH expression was subsequently analysed using a
HRP-conjugated monoclonal antibody against GAPDH on the same blot.

2.8. Statistical analysis
Differences in responses were analyzed using one-way or two-way ANOVA analyses with
Bonferroni adjustment, the two tailed student t-test or the paired student t-test. The statistical
significance level used was p<0.05.
3. Results

3.1. IFN-α and IFN-β inhibit IFN-γ induced CD64 and CD54 expression
We have previously noted that IFN-α inhibited the expression of CD54 and CD64 induced by 2.5 ng/ml of IFN-γ [13]. Little is known about the dose response effects of IFN-α/β and IFN-γ, which we studied here in detail. Furthermore, we determined whether IFN-β displays similar effects and whether high concentrations of IFN-γ could overcome these inhibitory effects. CD14+ monocytes were cultured for 18 h with various concentrations of IFN-γ in the presence or absence of IFN-α or IFN-β. Incubation of the CD14+ monocytes with high concentrations of IFN-γ gave a 6 fold enhancement in CD64 expression (Fig. 1A and 1B). Culturing the cells with IFN-α or IFN-β alone did not affect CD64 expression. Addition of IFN-α or IFN-β together with IFN-γ gave a dose dependent reduction of the IFN-γ induced CD64 expression (Fig. 1A and 1B). IFN-α and IFN-β reduced the expression of CD64, at all IFN-γ concentrations. The IFN-γ induced enhancement of CD54 expression was reduced by IFN-α and IFN-β in a similar way (Fig. 1C and 1D). The inhibition by IFN-α and IFN-β was similar (Fig. 1E) and IFN-γ concentrations up to 250 ng/ml could not overcome the inhibitory effect of IFN-α or IFN-β (Fig 1A-D).

3.2. IFN-α and IFN-β inhibit production of IL-12p40, IL-1β and TNF
IFN-α and IFN-β are known to inhibit IL-12p40 production. We wanted to determine the extent of this inhibition and whether other cytokines are also inhibited. For the establishment of an effective type I immune response the cytokines TNF and IL-1β play a pivotal role. The production of these cytokines can be induced via TLR stimulation and is greatly enhanced by IFN-γ. Therefore, we studied the effects of IFN-α and IFN-β on the production of IL-12p40 (the common subunit of IL-12 and IL-23), IL-1β and TNF by monocytes. CD14+ monocytes were stimulated with or without 1000 U/ml IFN-α or IFN-β and various concentrations of IFN-γ in the presence or absence of LPS. Incubation with one of the interferons alone did not induce cytokine production (data not shown). LPS induced the production of IL-12p40, IL-1β and TNF, which could be dose dependently enhanced by IFN-γ (Fig. 2 A, B, C). IFN-α and IFN-β strongly reduced the production of IL-12p40 induced by LPS alone or together with IFN-γ (Fig. 2A). The effect of IFN-γ on IL-1β production was also strongly reduced (Fig. 2B), while the effect of IFN-γ on TNF production was only partly reduced by IFN-α and IFN-β (Fig. 2C). Addition of high IFN-γ concentrations, up to 250 ng/ml, could not overcome the inhibitory effects of IFN-α or IFN-β on the LPS-induced IL-12p40, IL-1β and TNF production (Fig 2A, B, C). The extent of the inhibitory effects of IFN-α and IFN-β was compared in figure 2D. The inhibitory effect was significant for all three cytokines and was similar for IFN-α and IFN-β (Fig. 2D). The inhibitory effects of the IFN-α and IFN-β were not observed when LPS or IFN-γ were added 1 h prior to the addition of IFN-α (data not shown).
Besides LPS, which is a single TLR-ligand able to activate monocytes via TLR2 and TLR4, we investigated whether IFN-α and IFN-β could also inhibit the responses induced by Mycobacteria. Hereeto, we stimulated CD14+ monocytes with or without IFN-α or IFN-β and IFN-γ in the presence or absence of MTB. The MTB induced production of the three measured cytokines was reduced by IFN-α and IFN-β (Fig. 2E, F, G). The synergistic effect of IFN-γ on the MTB induced IL-12p40 and IL-1β production was blocked by IFN-α and IFN-β (Fig. 2E and 2F), while the TNF production was partially reduced by IFN-α and IFN-β (Fig. 2G).

Figure 1. Inhibition of IFN-γ induced CD54 and CD64 expression by IFN-α and IFN-β. CD14+ monocytes were stimulated with various concentrations of IFN-γ and IFN-α (A, B) or IFN-β (C, D). After 18 h the CD54 and CD64 expression was measured by FACS. Four donors were tested separately. One representative experiment is shown (A, B, C, D). The influence of 1000 U/ml of IFN-α on the CD64 and CD54 expression (E), induced by 250 ng/ml IFN-γ, is depicted as the percentage of inhibition by IFN-α. IFN-γ induced expression was set at 100% and expression in untreated cells was set at 0%. The mean +/- the standard deviation is displayed for four donors.
Figure 2. Inhibition of TLR-ligand and IFN-γ induced cytokine production by IFN-α and IFN-β. CD14+ monocytes were stimulated with 1 ng/ml LPS and various concentrations of IFN-γ with or without 1000 U/ml IFN-α or IFN-β. After 24h the production of IL-12p40 (A), IL-1β (B) and TNF (C) were determined by ELISA. Four donors were tested, one representative experiment is shown. (D) The influence of 1000 U/ml of IFN-α/β on cytokine production, induced by LPS and 250 ng/ml IFN-γ, is depicted as the percentage of inhibition by IFN-α/β. LPS/IFN-γ induced production was set at 100% and LPS induced production was set at 0%. The effect of IFN-α/β on mycobacterial stimulation (E-G) was determined by incubating CD14+ monocytes with 10 μg/ml MTB, with or without 2.5 ng/ml IFN-γ and with or without 1000 U/ml IFN-α or IFN-β. After 24h the production of IL-12p40 (E), IL-1β (F) and TNF (G) were determined by ELISA. Three donors were tested in triplo. One representative experiment is shown. Error bars represent the standard deviation. * p<0.01 (one-way ANOVA).
Figure 3. IFN-α elicits prolonged inhibitory effects on type I immune responses in vitro and in vivo. The duration of the inhibitory effects of IFN-α on type I immune response was studied in vitro (A, B). IFN-α was added to CD14⁺ monocytes 24 h, 4 h or 0 h prior to the addition of 2.5 ng/ml IFN-γ or medium. The cells were cultured for another 18 h before expression of CD64 (A) or CD54 (B) was analyzed. The results are displayed as the mean fluorescence +/- standard deviation of an experiment performed in triplo. One representative experiment out of three is shown. * significantly different from stimulation without IFN-α and ** significantly different pairs, p<0.05 (two-way ANOVA). To study the inhibitory effects of IFN-α in vivo (C), PBMCs from melanoma patients were obtained before and during treatment with IFN-α and cultured for 18 h with various concentrations of IFN-γ. Afterwards, CD54 expression of the CD14⁺ cells was analyzed by FACS, the relative mean fluorescence +/- standard deviation of four patients are shown (C). In addition, PBMCs from four patients and five controls were stimulated for 24 h with 10 ng/ml LPS or medium (D). IL-12p40 production in supernatants was analyzed by ELISA (D). For C * p<0.01 by one-way ANOVA and for D * p<0.05 (paired student t-test, unpaired student t-test was used for comparison with controls).
3.3. IFN-α elicits prolonged inhibition of type I immune responses in vitro and in vivo

IFN-α inhibits the IFN-γ responses when both cytokines are given together to the monocytes. It is not known how long the inhibitory effect of IFN-α lasts. We investigated whether IFN-α could still effectively inhibit type I immune responses until 24h after IFN-α stimulation. Therefore CD14+ monocytes were pre-cultured in the presence of IFN-α and subsequently stimulated with IFN-γ. IFN-α inhibited the IFN-γ induced CD64 expression when given together with IFN-γ and when given 4 h prior to IFN-γ to a similar extent (Fig. 3A). When IFN-α was given 24 h prior to the addition of IFN-γ the inhibition of CD64 expression was even stronger. IFN-α also displayed strong inhibitory effects on the induction of CD54 expression when given 4 h or 24 h prior to IFN-γ (Fig. 3B).

To study whether IFN-α also inhibits monocytes in vivo we analyzed the PBMCs of melanoma patients before and during IFN-α treatment. Before and after at least one week of daily IFN-α injections we determined the basal and IFN-γ inducible expression of CD54 and the LPS-induced IL-12p40 production. Basal expression of CD54 was significantly reduced during IFN-α treatment (Fig. 3C). IFN-γ could not enhance the CD54 expression to maximum levels when the patients received IFN-α treatment. At all IFN-γ concentrations the CD54 expression was significantly lower during IFN-α treatment. Due to the IFN-α treatment, the capacity of the PBMCs to produce IL-12p40 upon LPS stimulation was reduced (Fig. 3D). Unstimulated PBMCs did not produce any detectable IL-12p40 (data not shown). The IFN-γR1 expression of the PBMCs, before and during IFN-α treatment, was comparable (data not shown).

3.4. Downregulation of IFN-γR1 expression by IFN-α and IFN-β

IFN-γ binding to its receptor complex, consisting of two IFN-γR1 and two IFN–γR2 chains, results in signal transduction and subsequent downregulation of the cell surface expression of IFN-γR1. Little is known about the kinetics of IFN-γR downregulation in human monocytes. We investigated the kinetics of IFN-γR1 downregulation on CD14+ monocytes by IFN-γ in more detail and whether IFN-α and IFN-β could also induce IFN-γR1 downregulation, independent from IFN-γ. The cell surface expression of the IFN-γR1 receptor was, after a delay of 2 h, gradually downregulated by IFN-γ, was strongly reduced after 4 h (up to 77%), and was still remarkably reduced after 18 h of stimulation (Fig. 4A). The IFN-γR1 expression was gradually reduced at all IFN-α concentrations, also with a delay of 2 h after stimulation, and was most strongly reduced after 4 h (up to 75%). The IFN-γR1 expression was partly restored after 18 h of stimulation with IFN-α (Fig. 4A).

We next analyzed the effects of simultaneous stimulation with IFN-α and IFN-γ and whether a 4h or 24h pre-stimulation with IFN-α had an additive or synergistic effect on the IFN-γR1 downregulation induced by IFN-γ. We did not observe any additive effect of IFN-α and IFN-γ (Fig. 4B). Pre-stimulation of IFN-α did not influence the kinetics of the IFN-
γR1 downregulation by IFN-γ (Fig. 4B). IFN-β downregulated the IFN-γR1 expression in a similar manner (data not shown). As previously reported, cell surface expression of IFN-γR2 on monocytes could not be detected by FACS [25].

3.5. IFN-α signalling interferes with IFN-γ induced STAT1 phosphorylation
IFN-γ signalling occurs via the formation of STAT1 homodimers, while IFN-α signals via STAT1-STAT2 heterodimer complexes. The signalling of both interferons may influence each other through competition for STAT1. We examined the kinetics of STAT1 and STAT2 phosphorylation in CD14+ monocytes after addition of IFN-α and IFN-γ alone or together. STAT1 phosphorylation was quantified by FACS (Fig. 4C). There was only a very small additive effect in STAT1 phosphorylation after stimulation for 5 or 10 minutes with 0.25 ng/ml of IFN-γ and 100 U/ml of IFN-α, although maximum STAT1 phosphorylation was not reached, as illustrated by the use of a higher concentration of IFN-γ or by longer stimulation (15 minutes). The additive effect at 5 and 10 minutes was smaller than expected of a full additive effect (which can be calculated as the sum of the response induced by IFN-α and the response induced by IFN-γ). After 5, 10 and 15 minutes of stimulation with 2.5 ng/ml IFN-γ and 100 U/ml of IFN-α, no additive effects were observed (Fig. 4C).

Because no antibodies are available to quantify tyrosine phosphorylated STAT2 by FACS, we determined the STAT2 phosphorylation by western blot analysis. No STAT2 phosphorylation was observed in unstimulated cells. As expected, IFN-γ did not induce any detectable STAT2 phosphorylation. IFN-α clearly induced STAT2 phosphorylation, which was not changed in the presence of IFN-γ (Fig. 4D).

Surprisingly, hardly any additive effects of IFN-α and IFN-γ were observed for the STAT1 phosphorylation, and IFN-α induced the same STAT2 phosphorylation in the presence or absence of IFN-γ. We argue that there may be a considerable reduction of IFN-γ induced STAT1 homodimer formation due to competition between STAT2 and STAT1 for dimer formation with STAT1. To estimate the reduction of STAT1 homodimer formation we compared the amount of STAT1 phosphorylated by IFN-γ alone with the amount of STAT1 phosphorylated by the costimulation of IFN-γ and IFN-α minus the amount of STAT1 phosphorylated by IFN-α alone. In this way, we estimate that there may be a reduction in STAT1 homodimer formation of up to 17%, 44% and 58% after respectively 5, 10 and 15 minutes of stimulation. These calculated reductions are probably overestimated, because we presumed that all phosphorylated STAT1 dimerizes and because we cannot fully exclude that the IFN-α induced STAT2 phosphorylation is not slightly reduced in the presence of IFN-γ.
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**Figure 4.** IFN-α downregulates IFN-γR1 expression and interferes in the IFN-γ induced signal transduction. IFN-γR1 expression on CD14⁺ monocytes was measured by FACS after stimulation with medium, with 2.5 ng/ml IFN-γ or with various concentrations of IFN-α (A). Mean fluorescence of unlabeled monocytes was set at 0% and mean fluorescence of labelled untreated monocytes was set at 100%. IFN-γR1 expression was calculated as percentage of the controls. The mean +/- the standard deviation of three donors is shown. * significantly different from the untreated control p<0.05 (two-way ANOVA). The effect of costimulation with 1000 U/ml IFN-α and the effect of prestimulation with 1000 U/ml IFN-α on the IFN-γ induced IFN-γR1 downregulation was tested in triplo for two donors (B). The measured IFN-γR1 expression at 0 h was set at 100%. The mean +/- the standard deviation is shown. * significantly different from the expression measured at 0 h p<0.05 (two-way ANOVA). The signal transduction was determined by measuring the STAT1 and STAT2 phosphorylation (C and D). CD14⁺ monocytes were stimulated with 0.25 ng/ml (C, D) or 2.5 ng/ml (A) IFN-γ and 100 U/ml of IFN-α (A, B). At various time points the STAT1 tyrosine phosphorylation was determined by FACS (A). The mean fluorescence +/- the standard deviation of three individual measurements is shown. The amount of tyrosine phosphorylated STAT2 and GAPDH was determined by western blot (B), one representative experiment out of three is shown.
3.6. Monocytes show basal PRMT1 expression and inducible PIAS1 expression

IFN-α could potentially inhibit IFN-γ responses via activation of PRMT1 and/or via induction of factors that negatively influence the JAK/STAT pathway, such as PIAS1. To establish whether these factors play a role in the inhibitory effects of IFN-α we investigated the basal and inducible expression of PRMT1 and PIAS1 in monocytes. CD14⁺ monocytes were stimulated for 18 h with combinations of IFN-α, IFN-γ and LPS. Monocytes showed basal expression of PRMT1 which was not further enhanced by the stimuli (Fig. 5A). A small amount of PIAS1 was expressed in unstimulated monocytes and could be enhanced by either IFN-α, IFN-γ or LPS alone or by combinations of these stimuli (Fig. 5A). Next, we determined the kinetics of PIAS1 upregulation by IFN-α or IFN-γ. Both interferons induced PIAS1 expression, relatively late, after more than 8 h of stimulation (Fig. 5B).

3.7. Inhibition of PRMTs reduces the inhibitory effects of IFN-α

IFN-α could potentially activate PRMT1, which in turn can influence the expression of immune genes via methylation of various regulatory proteins. With the use of a competitive PRMT inhibitor, AMI1 [26], we investigated the role of PRMTs in the inhibitory effects of IFN-α.

Figure 5. Basal expression of PRMT1 and interferon inducible PIAS1 expression. The expression of PRMT1, PIAS1 and GAPDH in CD14⁺ monocytes was analyzed by western blot after 18 h of stimulation with medium, 1000 U/ml IFN-α, 25 ng/ml IFN-γ, 1 ng/ml of LPS or with combinations of the stimuli (A). The kinetics of PIAS1 expression was determined after stimulation with 1000 U/ml IFN-α or 25 ng/ml IFN-γ (B).
First, we determined the role of PRMTs in the regulation of CD54 and CD64 expression. CD14+ monocytes were pre-incubated with AMI1 and cultured in the presence of IFN-γ and various concentrations of IFN-α. IFN-α reduced the IFN-γ upregulated CD54 expression (Fig. 6A). Pre-incubation with AMI1 resulted in a significantly smaller reduction (Fig. 6C). In contrast, IFN-γ induced expression of CD64 was already somewhat reduced by AMI1 itself (Fig. 6B), while AMI1 had no influence on the inhibitory effect of IFN-α on CD64 expression (Fig. 6D).

**Figure 6. Inhibition of PRMTs reduces the IFN-α effect on IFN-γ induced CD54 and CD64 expression.** The PRMT inhibitor AMI1 was added in a concentration of 200 µM to CD14+ monocytes cultures 1 h before the addition of 2.5 ng/ml IFN-γ and various concentrations of IFN-α. The induction of CD54 (A) and CD64 (B) was measured by FACS. The fluorescence of unlabelled cells was set at 0% and the fluorescence of the IFN-γ stimulated and CD54 or CD64 labelled cells was set at 100%. The results of one donor tested in triplo is shown, error bars represent the standard deviation (A, B). To indicate significant effects of IFN-α, the results of three donors were sampled (C, D). Therefore, the inhibition of IFN-α on the IFN-γ mediated CD54 (C) and CD64 (D) expression was calculated as the percentage of inhibition: IFN-γ induced expression was set at 0% and the basal expression was set at 100%. The mean and standard deviation of three donors, each tested in triplo, is shown (C,D). p<0.05 (two-way ANOVA), n.s. not significant.
Figure 7. Inhibition of PRMTs reduces the effect of IFN-α on IFN-γ induced cytokine production. CD14+ monocytes were pre-incubated for 1 h with or without 200 μM of the PRMT inhibitor AMI1. Subsequently the cells were stimulated for 24 h with 1 ng/ml LPS with or without 2.5 ng/ml IFN-γ and various concentrations of IFN-α. After 24 h the production of IL-12p40 (A, D), IL-1β (B, E) and TNF (C, F) were measured by ELISA. The results of one out of three donors are shown (A, B, C). To indicate significant effects of IFN-α, the results of three donors were sampled (D, E, F). The inhibition by IFN-α on the LPS/IFN-γ induced cytokine production was calculated as percentage of inhibition (D, E, F). The LPS/IFN-γ induced cytokine production was set at 0% and the LPS-induced production was set at 100%. The mean and the standard deviation of three donors is shown (D, E, F). AMI1 alone did not induce cytokine production (data not shown). p<0.05 (two-way ANOVA), n.s. not significant.
Next, we analysed the influence of AMI1 on LPS and IFN-γ induced cytokine production. CD14+ monocytes were pre-incubated with AMI1 and then stimulated with IFN-γ and various concentrations of IFN-α in the presence of LPS. AMI1 alone did not induce any cytokine production (data not shown). AMI1 pre-incubation already resulted in a 2 fold enhanced LPS/IFN-γ-induced IL-12p40 production, without IFN-α (Fig. 7A). AMI1 had little influence on the effects of IFN-α on the LPS and IFN-γ induced IL-12p40 production (Fig. 7A, D). IFN-α reduced the LPS/IFN-γ induced IL-12p40 production. Pre-incubation with AMI1 resulted in a similar reduction of IL-12p40 by IFN-α (Fig. 7D). IFN-α reduced the LPS/IFN-γ induced IL-1β and TNF production, which was for both cytokines significantly less after pre-incubation with AMI1 (Fig. 7B, C, E, F).

4. Discussion

This report demonstrates that IFN-α/β strongly inhibit the type I cytokine production and the IFN-γ responsiveness of human monocytes, probably via various mechanisms. Previously, we and others reported that exposure of monocytes to IFN-α/β leads to a reduction of IL-12p40 production and IFN-γ responsiveness [12, 13]. We now demonstrate that IFN-α and IFN-β have comparable strong inhibiting effects on human monocytes and that exposure to high concentrations of IFN-α could not counterbalance the inhibitory effects of either IFN-α or IFN-β. Furthermore, our results indicate that IFN-α interferes with the IFN-γ responsiveness, early and late after stimulation. To explore the mechanisms of inhibition we studied the kinetics of the IFN-α induced IFN-γR1 downregulation showed that the downregulation was transient and occurred with two hours delay after IFN-α stimulation, indicating that other mechanisms are involved in the direct and late inhibitory effects. Indeed, we found some additional evidence that IFN-α directly interferes with the IFN-γ mediated signalling and that IFN-α may, at least in part, inhibit the production of IL-1β and TNF via activation of a PRMT, probably PRMT1. Furthermore, we show that IFN-α induces PIAS1, a negative regulator of STAT1 mediated transcription, which indicates that a PRMT1/PIAS1 pathway may be involved as well.

Both IFN-α and IFN-β reduced the IFN-γ induced CD64 and CD54 expression as well as the LPS and IFN-γ induced IL-12p40, IL-1β and TNF production. IFN-α and IFN-β showed the strongest inhibition on the LPS and MTB induced production of IL-12p40, the shared subunit of IL-12 and IL-23. Both IL-12 and IL-23 play an important role in the mounting of type I immune responses. IL-12 is known to stimulate IFN-γ production, Th1 polarization and expansion of T-cells subsets. IL-23 stimulates NK-like T cells to produce IFN-γ [27]. In turn, IFN-γ modulates monocytes in their type I immune functions, which can also be
antagonized by IFN-α. These inhibitory effects on monocytes occurred in vitro for at least 24 h after IFN-α stimulation and endured in vivo in IFN-α treated patients. The severe impact of IFN-α on type I immunity, the importance of IFN-γ in the control of mycobacterial diseases, the ability of virulent mycobacterial strains to induce IFN-α [5] and the finding of a typical IFN-α transcript signature in the blood cells of TB patients [2, 3] suggest an important role of IFN-α/β in the immunopathogenesis of *Mycobacterium tuberculosis* infections.

One mechanism through which IFN-α/β inhibits IFN-γ signalling may be downregulation of IFN-γR1 cell surface expression. IFN-γR downregulation by IFN-α/β was observed in mice after *Listeria monocytogenes* infection. Receptor down-regulation was found to be responsible for the IFN-γ unresponsiveness of monocytes after *Listeria* encounter [28]. In human macrophages, IFN-γR1 expression was also found to be reduced after incubation with IFN-α [29]. Our kinetic studies show that IFN-γR1 downregulation occurred only after two hours of stimulation with IFN-α and appeared to be transient. Hence, the IFN-γR1 downregulation can not explain the inhibitory effects of IFN-α we observed when IFN-α was given together with or 24 h before IFN-γ in vitro or when given to patients in vivo. Thus, our results indicate that other mechanisms are also involved to achieve direct and enduring opposing effects on the type I immune responses of monocytes.

The fact that IFN-α/β can inhibit IFN-γ mediated responses when given to monocytes simultaneous with IFN-γ indicates that these interferons can also interfere directly with IFN-γ mediated signalling. Indeed, by studying the kinetics of STAT1 and STAT2 phosphorylation we found some evidence that there is a direct negative effect of IFN-α on IFN-γ signalling. The amount of phosphorylated STAT1 induced by IFN-γ signalling is reduced in the presence of IFN-α. We reason that this reduction in STAT1 phosphorylation may result in a reduction of STAT1 homodimer formation of maximal 58% and could thereby account for a severe loss of IFN-γ responsiveness. Previous investigations with human fetal astrocytes [30] and murine macrophages [31], indicate also that IFN-β is involved in IFN-γ signalling by reducing the amount of STAT 1 homodimers bound to DNA [30] and influencing the STAT1 dephosphorylation [31]. It would be interesting to perform similar investigations with human monocytes and other phagocytes with ChIP analysis, using DNA probes coding for different GAS sites, in order to quantify the exact amounts of STAT1 homodimers, ISGF3 complexes and bioactive PIAS1 in the nucleus.

Another mechanism whereby IFN-α may oppose type I immune modulation is the activation of PRMTs. PRMT1 can associate with the intracellular part of IFN-αR1 and can be activated by IFN-α [19]. We show that PRMT1 is expressed in unstimulated human monocytes. And with the use of an inhibitor of PRMTs we revealed that only some of the inhibitory effects of IFN-α could be ascribed to a PRMT pathway. This was most prominent for the inhibition of IL-1β and TNF production, but was also observed for CD54 expression.
Further extensive investigation is needed to reveal which PRMT and which PRMT targets are involved.

PRMT1 is, for instance, able to methylate PIAS1, a negative regulator of the transcriptional activity of STAT1 [22, 23]. We show that expression of PIAS1 is induced relatively late after stimulation with IFN-γ, apparently as a negative feedback on IFN-γ signalling. IFN-α also induces PIAS1, with similar kinetics. The relatively late induction of PIAS1 may partly explain the fact that 24 h of pre-stimulation of monocytes with IFN-α results in a strong inhibition of the IFN-γ responsiveness in vitro, and why a lasting inhibitory effect on type I immunity could be observed in IFN-α treated patients.

Other mechanisms could also be involved. Gene transcription may be indirectly regulated by transcription factors other than STAT1 and STAT2. For example, the induction of the transcription factor IRF-8 by IFN-γ can be antagonized by IFN-α [18]. Furthermore, we speculate that there could be negative interference of ISGF3 with transcriptional activity. This may be the case with CD64 transcription. Despite the fact that the promoter of CD64 contains an ISRE-like element, which is a putative ISGF3 binding site, we showed that IFN-α does not induce CD64 expression. In fact, IFN-γ induced CD64 expression was inhibited by IFN-α. This suggests that the inhibitory effects of IFN-α may be due to a partial or complete block of transcriptional activity as a result of binding of ISGF3 to the promoter of CD64.

Taken together, IFN-α stimulation of monocytes results in a broad range of opposing effects on type I immunity, which may be established by various mechanisms. Some of these effects sustain for at least 24 h after IFN-α stimulation, in vitro as well as in vivo, indicating that IFN-α is an important negative regulator of the type I immune response. These effects may explain the enhanced susceptibility to bacterial pneumonia just after influenza infection [32]. Similarly, IFN-α/β induced by intracellular pathogens, such as Listeria monocytogenes [28] or virulent Mycobacterium tuberculosis [5] strains, can dampen the formation of an effective type I immune response. The benefit of the inhibitory effects of IFN-α for the host is not really understood. We speculate that a type I immune response during the early phase of viral infections may otherwise easily escalate in hyperinflammatory reactions.

Because IFN-α induces strong opposing effects on type I immune responses this should be taken into account in the treatment of certain patients. First, we recently reported that IFN-α treatment of IFN-γR1 deficient patients is not advisable [13]. Our present data further strengthen the advice not to use IFN-α in patients lacking Th1 immunity. Second, when considering IFN-α treatment in other types of patients, clinicians should be aware of the type I immune inhibiting effects. Several case reports of hepatitis patients who developed Tuberculosis during IFN-α treatment [33-35] indicate that perhaps patients from countries with high Tuberculosis prevalence should be screened for latent Tuberculosis prior to IFN-α treatment. Third, although cancer patients receiving IFN-α treatment to support T cell therapy may respond well to the therapy, it is possible that the inhibition of the type I
immune responses hampers the therapy to some extent. Fourth, in the development of *M. tuberculosis* infections IFN-α/β appear to play an important role [2], the source of this IFN-α/β is however still unclear. IFN-α/β may be produced by infected monocytes and/or during viral infection, allowing *M. tuberculosis* to take advantage of the situation and develop into an active infection. Thus, co-infections of *M. tuberculosis* with common types of viruses may aggravate the course of *M. tuberculosis* infections (R.A. de Paus et al, submitted for publication).

In conclusion, IFN-α and IFN-β both strongly inhibit monocytes in their capacity to produce type I cytokines and to respond to IFN-γ. The impact of IFN-α and IFN-β may indicate a major role of these interferons in the pathogenesis of infections with *M. tuberculosis* and other intracellular bacterial pathogens which are able to induce IFN-α/β. Our data provide novel insights into the mechanisms of the inhibitory effects. These effects may be achieved via various mechanisms, such as, a decreased IFN-γ signalling, a transient decrease in IFN-γR1 expression, activation of PRMTs and the induction of PIAS1. Further detailed investigations should dissect the exact contributions of each of these putative mechanisms.
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References


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