IFNα induces a preferential long-lasting expression of MHC class I in human pancreatic beta cells

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Abstract

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**Aim/hypothesis** IFNα, a cytokine expressed in human islets from type 1 diabetes patients, plays a key role in the pathogenesis of diabetes by up-regulating inflammation, endoplasmic reticulum (ER) stress and MHC class I overexpression, three hallmarks of islet histology in early type 1 diabetes. We presently tested whether expression of these mediators of beta cell loss is reversible upon IFNα withdrawal or IFNα pathway inhibition.

**Methods** IFNα-induced MHC class I overexpression, ER stress and inflammation were evaluated by flow cytometry, immunofluorescence and RT-PCR in human EndoC-βH1 cells or human islets exposed to IFNα with or without the presence of JAK inhibitors. Protein expression was evaluated by western blot.

**Results** IFNα-induced expression of inflammatory and ER stress markers returned to baseline after 24-48 h following cytokine removal. By contrast, MHC class I overexpression at the cell surface persisted for at least 7 days. Treatment with JAK inhibitors, added together with IFNα, prevented MHC class I overexpression, but when added 24 h after IFNα exposure these inhibitors failed to accelerate MHC class I return to baseline.

**Conclusion/interpretation** IFNα mediates a long-lasting and preferential MHC class I overexpression in human beta cells, which is not affected by the subsequent addition of JAK inhibitors. These observations suggest that IFNα-stimulated long-lasting MHC class I expression may amplify beta cell antigen presentation during the early phases of type 1 diabetes and that IFNα inhibitors might need to be used at very early stages of the disease to be effective.

**Keywords:** Type 1 diabetes, IFNα, MHC class I, pancreatic beta cells, pancreatic islets, JAK inhibitors

**Abbreviations:**
- BIP  Binding immunoglobulin protein
- CHOP  C/EBP homologous protein
- CHX  Cycloheximide
- CXCL10  C-X-C motif chemokine ligand 10
- ER  Endoplasmic reticulum
- HO  Hoechst 33342
- JAK  Janus kinase
MX1  MX dynamin like GTPase 1
STAT  Signal transducer and activator of transcription
TYK2  Tyrosine kinase 2

**Introduction**

The overexpression of major histocompatibility complex (MHC) class I in pancreatic islets, probably secondary to local interferon-α (IFNα) production, is a key feature in the pathogenesis of type 1 diabetes [1, 2]. The primary function of MHC class I is to transport intracellular (endogenous or viral) peptides to the cell surface, where they are presented and recognized by cytotoxic CD8⁺ T cells, leading to killing of the antigen-expressing cells or, in some cases, to development of tolerance [3].

We have recently shown that IFNα induces MHC class I overexpression, inflammation and endoplasmic reticulum (ER) stress in human beta cells. These effects are mediated via activation of the Janus kinase (JAK-TYK2)/STAT pathway [4], with a key role for the type 1 diabetes candidate gene TYK2, whose encoded protein phosphorylates and activates STATs [4, 5]. JAK/TYK2 inhibitors have been recently approved for the treatment of other autoimmune diseases, such as rheumatoid arthritis [6].

To evaluate the possibility of targeting the IFNα pathway for the treatment of type 1 diabetes, we presently tested whether the effects of IFNα are reversible upon cytokine withdrawal or IFNα pathway inhibition. We present evidence that IFNα promotes a specific and long-lasting MHC class I overexpression in human beta cells. Moreover, while exposure of human beta cells to IFNα in the presence of the JAK inhibitors ruxolitinib and cerdulatinib prevents IFNα-induced expression of MHC class I and other stress markers, these inhibitors fail to accelerate MHC class I return to baseline if added 24 h after IFNα exposure. These results suggest that IFNα-stimulated long-lasting MHC class I expression may contribute to prolonged beta cell antigen presentation during early steps of type 1 diabetes.

**Methods**

**Culture of human EndoC-βH1 cells and human islets, and cell treatments**

The human beta cell line EndoC-βH1 (kindly provided by Dr. R. Scharffmann, University of Paris, France) was cultured in Matrigel-fibronectin-coated plates as described [4]. These cells
are free from mycoplasma infection, as evaluated by MycoAlert Mycoplasma Detection kit (Lonza, Basel, Switzerland).

Isolation of human islets from 3 non-diabetic organ donors (ESM Table 1) was performed in accordance with the local Ethical Committee in Pisa, Italy. After arrival in Brussels, islets were dispersed and cultured as in [4]. All experiments shown with EndoC-βH1 cells or human islet cells (indicated as “n” in the figures) refer to independent biological data (i.e. using EndoC-βH1 cells from different passages or human islets from different donors). Where indicated, cells were treated with human IFNα (PeproTech Inc., Rocky Hill, NJ) 20 or 1000 U/ml [4]. Cells were treated with ruxolitinib (kindly provided by Calibr, CA, USA), cerdulatinib (Selleckchem, Germany), Bayer-18 (Synkinase, UK), or cycloheximide (Sigma-Aldrich, Germany) as indicated.

**Flow cytometry and immunofluorescence**

EndoC-βH1 cells were plated in 24-well plates (150,000 cells/well). After treatment, cells were incubated with mouse anti-MHC class I antibody (W6/32) (1:1000) for 2 h and subsequently with a BV421-conjugated secondary antibody before analysis by flow cytometry (FacsCanto; BD Biosciences, CA, USA) as described [4]. The cellular populations were separated based on size and granularity and further analysed for BV421 fluorescence.

Immunofluorescence was performed as previously described [4], using mouse anti-MHC class I (W6/32) (1:1000) and guinea pig anti-insulin antibodies (1:250), Hoechst (HO) and Alexa Fluor-conjugated secondary antibodies (ESM Table 2).

**mRNA extraction and real-time PCR**

EndoC-βH1 cells were plated in 96-well plates (45,000 cells/well). After treatment, poly(A)^+ mRNA was isolated using the Dynabeads mRNA DIRECT kit (Invitrogen) following the manufacturer’s instructions, and reverse transcribed as described [5]. Quantification by real-time PCR was carried out using SYBR Green. Gene expression values were corrected by the housekeeping gene β-actin, as its expression is not affected by the conditions used in this study [5]. The primers used are listed in ESM Table 3.

**Statistical analysis**

Data are expressed as means ± SEM. A significant difference between experimental conditions was assessed by one-way or two-ways ANOVA followed by paired or unpaired t test with Bonferroni correction using the GraphPad Prism program. Results with \( p<0.05 \) were considered statistically significant.
Results
IFNα, tested at two concentrations (1000 and 20 U/ml), strongly induced the expression of MHC class I (Fig. 1a-c and ESM Fig. 1a-c), the chemokine C-X-C motif chemokine ligand 10 (CXCL10), the antiviral MX dynamin like GTPase 1 (MX1) (Fig. 1d, f and ESM Fig. 1d, e), and the ER stress markers C/EBP homologous protein (CHOP) and the binding immunoglobulin protein BIP (Fig. 1g, h and ESM Fig. 1f, g) in EndoC-βH1 cells. When IFNα was removed from the medium (“wash”) and the cells cultured in its absence for subsequent periods of 24 h to 7 days, MHC class I protein and, to a less extent, mRNA remained overexpressed for at least 7 days (Fig. 1a-c and ESM Fig. 1a-c), while the inflammatory markers CXCL10 and MX1 (Fig. 1d, f and ESM Fig. 1d, e) and the ER stress markers CHOP and BIP (Fig. 1g h and ESM Fig. 1f, g) started to decrease already by 24-48 h. CXCL10 secretion to the medium, as measured by ELISA, also decreased by 24 h, returning to near basal (control) levels by 72 h (Fig. 1e). Importantly, IFNα-mediated MHC class I overexpression also persisted for at least 7 days in dispersed human islets (Fig. 2).

Pre-treatment with the JAK inhibitors ruxolitinib and cerdulatinib prevented IFNα-induced HLA-ABC, CXCL10, MX1 and CHOP expression in a dose-dependent manner (ESM Fig. 2a-h). These JAK inhibitors also prevented IFNα-induced CXCL10 secretion (ESM Fig. 2m). On the other hand, the “TYK2 inhibitor” Bayer-18 showed no effect on IFNα-induced gene expression (ESM Fig. 2i-l) and therefore was not further used. We also evaluated the effect of Bayer-18 in two other cell lines (HeLa and PANC-1) and again fail to observe inhibition of IFNα-induced MHC class I expression (data not shown). This unexpected observation emphasizes the need to validate in human beta cells and other cell types the different JAK/TYK2 inhibitors, ahead of future clinical trials. Despite their ability to prevent IFNα signalling, ruxolitinib and cerdulatinib did not accelerate MHC class I return to baseline when added after IFNα stimulation and its subsequent removal (ESM Fig. 3), suggesting that continuous IFNα signalling is not necessary for the long-lasting MHC class I overexpression observed in human beta cells.

The protein synthesis inhibitor cycloheximide (CHX) significantly reduced MHC class I basal expression, while it did not affect IFNα-induced MHC class I expression over 16 h (ESM Fig. 4a, b). After 48 h in the continuous presence of CHX, IFNα-induced MHC class I overexpression remained unchanged and similar to non-CHX-treated cells (data not shown). These results suggest that IFNα both induces a marked MHC class I overexpression and
stabilizes the protein at the cell surface. Of note, CHX decreased β-catenin, β-actin, and BIP expression over time, confirming the efficiency of the treatment (ESM Fig. 4c, d).

**Discussion**

MHC class I overexpression is induced by proinflammatory cytokines, such as IFNα [2] and IFNγ [1], in human islets from type 1 diabetes patients. Besides inducing MHC class I expression, IFNα also induces human beta cell ER stress and production of chemokines [4], suggesting that this cytokine is a key player in the early stages of human type 1 diabetes and in the transition between innate and adaptive immune responses. We presently show that MHC class I remains overexpressed at the cell surface of beta cells even after 7 days of IFNα withdrawal, while markers of inflammation and ER stress rapidly return to baseline. When added together with IFNα, JAK inhibitors prevent these effects of the cytokine, but fail to accelerate the return to baseline if added 24 h after IFNα stimulation, suggesting that following beta cell exposure to the cytokine a continuous IFNα stimulation is not required for the persistent MHC class I expression at the cell surface. These findings rise the possibility that IFNα-stimulated MHC class I expression, together with other inflammatory mediators, will amplify beta cell antigen presentation during the early phases of type 1 diabetes and suggest that IFNα inhibitors might need to be used at very early stages of the disease to be effective. These findings raise also the issue on the best “window of opportunity” for an eventual use of JAK inhibitors as therapeutic agents in T1D. Recent studies indicate that expression of a type I IFN response signature in circulating cells actually precedes the detection of autoantibodies in children at risk for T1D [7, 8]. If detection of this signature is shown to have accurate predictive power and becomes part of the routine follow up for at risk children, its presence could be a good moment for intervention. Alternatively, normoglycaemic children positive for two or more autoantibodies could be considered, but it remains to be seen whether this will allow reversion of the putative local manifestations of activated innate/adaptive immunity.

It is noteworthy that IFNα not only induces a massive overexpression of MHC class I but also seems to stabilize the protein at the cell surface of human beta cells (present data). In line with this, IFNγ induces both MHC class I expression and stability at the cell surface in murine myeloblast cells [9]. Ligand-free MHC molecules that reach the cell surface are rapidly degraded [10]. Thus, the presently observed long-lasting maintenance of MHC class I
expression at the human beta cell surface may be due to the parallel abundance of antigenic peptides generated in response to IFNα [11].

What is the “biological meaning” of this long-lasting IFNα-induced MHC class I mRNA and protein expression in human beta cells, while other markers of beta cell stress – including chemokines – return to baseline already by 24 h? A constant expression of MHC class I complexes in the absence of co-stimulation might be a mechanism to induce immune tolerance rather than immunity [12]. It is thus conceivable that, following a short local innate immune response (for instance, following brief “danger signals” provided by a limited viral infection or exposure to nuclear debris from dying cells), the transitory IFNα stimulation will lead to prolonged MHC class I expression in the absence of parallel chemokine production or expression of co-stimulatory molecules, favouring the switch from immunity to immune tolerance. Another possibility is that the prolonged expression of MHC class I on the target cells will enable the immune system to efficiently detect and delete beta cells expressing foreign peptides (e.g. cells with low or early viral infection), thus preventing a second wave of infection. It is conceivable that the role for the observed prolonged MHC class I expression in beta cells is context dependent, and additional studies are required to clarify this issue.

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Duality of interest
No potential conflicts of interest relevant to this article were reported.

Contribution statement
ACdB and RSS contributed to the original idea and the design of the experiments, researched data, contributed to discussion, and wrote, revised, and edited the manuscript. LM, M.L.C., Lo. M. and PM researched data and revised and edited the manuscript. RGM contributed to the original idea, experimental design and interpretation of the experiments. DLE contributed to the original idea and the design and interpretation of the experiments, contributed to discussion, and wrote, revised and edited the manuscript. All authors have read and approved the manuscript, and gave informed consent. ACdB and DLE are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References

**Figure legends**

**Figure 1. IFNα induces a specific and long-lasting MHC class I overexpression in EndoC-βH1 cells.**

EndoC-βH1 cells were left untreated (NT, black bars) or treated with IFNα (white bars; 1000 U/ml) for 24 h. Afterwards, culture medium was changed to remove IFNα (“wash”) and the cells were cultured in the absence of IFNα for 24 h, 48 h, 72 h, 96 h, or 7 days (grey bars). (a, b) MHC class I protein expression was measured by FACS. The percentage of positive cells (a) and the mean of fluorescence intensity (expressed as fold-change in MFI relative to the untreated sample) (b) were quantified. Results are means ± SEM of 4-18 independent measurements per condition (n=18 for NT and IFNα, and n=4-6 for the other conditions). mRNA expression of HLA-ABC (c), CXCL10 (d), MX1 (f), CHOP (g) and BIP (h) was analysed by RT-PCR, normalised by β-actin and then by the highest value of each experiment considered as 1. Results are means ± SEM of 3-9 independent experiments (i.e. using cells from different passages) per condition (n=9 for NT and IFNα, and n=3-5 for the other conditions). CXCL10 protein secretion to the supernatant was determined by ELISA (e). Results are means ± SEM of 6 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs NT; †p<0.05, ††p<0.01 and †††p<0.001 vs IFNα, as indicated by bars (one way ANOVA).

**Figure 2. IFNα induces a long-lasting MHC class I overexpression in dispersed human islets.**

Dispersed human islets were left untreated or treated with IFNα (1000 U/ml) for 24 h. Afterwards, culture medium was changed to remove IFNα (“wash”) and the cells were cultured in the absence of IFNα for 48 h or 7 days. Immunocytochemistry (ICC) analysis of MHC class I (red), insulin (green) and HO (blue) was performed to analyse MHC class I expression in 3 independent human islet preparations (magnification x40).
Figure 1

(a) MHC class I positive cells (%)
(b) MHC class I mean fluorescence intensity (fold)
(c) HLA-ABC mRNA (fold)
(d) CXCL10 mRNA (fold)
(e) CXCL10 (pg/ml)
(f) MX1 mRNA (fold)
(g) CHOP mRNA (fold)
(h) BIP mRNA (fold)
Figure 2

<table>
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ESM methods

Study design

For each recovery experiment (flow cytometry and gene expression), a condition “wash” - where cells were transitorily exposed to IFNα, followed by medium change (“wash”) - was compared to a negative control condition (not treated) and a positive control condition (IFNα 24 h; cells studied at the end of this period). We first evaluated the impact of “wash 24 h” (i.e. cells studied 24 h after removal of IFNα) on MHC class I expression and, observing the surprising finding that MHC class I remained overexpressed, progressively increased the period of incubation without IFNα to 48 h, 72 h, 96 h and 7 days until a significant difference with the positive control was observed. At least 3 independent experiments with respective controls (i.e. biologically different samples) were performed for each time point evaluated, therefore increasing the “n” for the controls. Since the control values were similar in the different experiments, they are presented as pooled results in the different figures.

ELISA

The CXCL10 release to the supernatant (by 45,000 cells/200 µl; culture time as indicated in the legend for the figures) was determined by enzyme-linked immunosorbent assay (Quantikine ELISA kit, R&D Systems, Minneapolis, MN, USA).

Western blot analysis

EndoC-βH1 cells were plated in 96-well plates (45,000 cells/well). After treatment, cells were washed with cold PBS and lysed using Laemmlı buffer. Immunoblot analyses were performed using antibodies against BIP, β-catenin (both at 1:1000 dilution), and β-actin (1:5000). Horseradish peroxidase-coupled antibodies were used as secondary antibodies (1:5000). Immunoreactive bands were detected as described using the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) and ChemiDoc XRS+ (Bio-Rad), and quantified with the Image Studio Lite v5.2 software (LI-COR Biosciences). A list of the antibodies used herein are described in ESM Table 2.
ESM Table 1. Characteristics of the human donors used in the present study.

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<th>Gender</th>
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<th>Cause of death</th>
<th>Proportion of beta cells in the preparation (%)</th>
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M (male); F (female); BMI (body mass index)
ESM Table 2. Antibodies used in the present study.

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Antibodies have been previously validated and used by our group [1].
**ESM Table 3. Primers used in the present study.**

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ESM Figure 1. A low dose of IFNα also induces a long-lasting MHC class I expression in EndoC-βH1 cells.

EndoC-βH1 cells were left untreated (NT, black bars) or treated with IFNα (white bars; 20 U/ml) for 24 h. The medium was then changed to remove IFNα (“wash”) and the cells were cultured in the absence of IFNα for subsequent periods of 24 h, 48 h, 72 h, 96 h or 7 days (grey bars). (a, b) MHC class I protein expression was measured by FACS. The percentage of positive cells (a) and the mean of fluorescence intensity (expressed as fold-change in MFI relative to the untreated sample) (b) were quantified. Results are means ± SEM of 3-11 independent measurements per condition (n=11 for NT and IFNα, and n=3-5 for the other conditions). mRNA expression of HLA-ABC (c), CXCL10 (d), MX1 (e), CHOP (f) and BIP (g) was analysed by RT-PCR, normalised by β-actin and then by the highest value of each experiment considered as 1. Results are means ± SEM of 2-8 independent measurements per condition (n=8 for NT and IFNα, and n=2-4 for the other conditions). **p<0.01 and ***p<0.001 vs NT; †p<0.05, ††p<0.01 and †††p<0.001 vs IFNα, as indicated by bars (one way ANOVA).
ESM Figure 2. JAK inhibitors block IFNα-induced gene expression in EndoC-βH1 cells in a dose-dependent manner.
EndoC-βH1 cells were left untreated or pre-treated with the indicated JAK inhibitor concentrations (100, 500, 1000 or 4000 nmol/l) for 2 h. Afterwards, cells were left untreated (black bars), treated with IFNα alone (white bars; 1000 U/ml) or with IFNα in presence of ruxolitinib (a-d, m), cerdulatinib (e-h, m) or Bayer-18 (i-l) (grey scale bars) for 24 h. mRNA expression of HLA-ABC (a, e, i), CXCL10 (b, f, j), MX1 (c, g, k) and CHOP (d, h, l) was analysed by RT-PCR, normalised by β-actin and then by the highest value of each experiment considered as 1. Results are means ± SEM of 3-9 independent experiments. CXCL10 protein secretion to the supernatant was determined by ELISA (e) after IFNα exposure for 24h in the presence or not of ruxolitinib (4000 nmol/l, light grey bar) or cerdulatinib (4000 nmol/l, dark grey bar). Results are means ± SEM of 3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs NT; ††p<0.01 and †††p<0.001 vs IFNα, as indicated by bars (one way ANOVA).

ESM Figure 3. Addition of JAK inhibitors after IFNα treatment does not accelerate MHC class I return to baseline.
EndoC-βH1 cells were left untreated (NT, black bars) or treated with IFNα (white bars; 1000 U/ml) for 24 h. Afterwards, culture medium was changed to remove IFNα and the cells were cultured in the absence of inhibitor (dark-grey bars; IFNα wash) or in the presence of ruxolitinib (light-grey bars) or cerdulatinib (stripped bars) (4 µmol/l) for 24 h, 48 h, 72 h or 7 days. (a, b) MHC class I protein expression was measured by FACS. The percentage of positive cells (a) and the mean of fluorescence intensity (b) were quantified. Results are means ± SEM of 3-20 independent experiments (n=20 for NT and IFNα, and n=3-5 for the other conditions). (c) mRNA expression of HLA-ABC was analysed by RT-PCR, normalised by β-actin and then by the highest value of each experiment considered as 1. Results are means ± SEM of 3-15 independent experiments (n=15 for NT and IFNα, and n=3-6 for the other conditions). **p<0.01 and ***p<0.001 vs NT; †p<0.05 vs IFNα wash and the same time point, as indicated by a bar (two ways ANOVA).

ESM Figure 4. IFNα stabilizes MHC class I proteins at the cell surface.
(a-b) EndoC-βH1 cells were left untreated (NT) or treated with IFNα (1000 U/ml) for 24 h. Afterwards, the medium was changed to remove IFNα and the cells were left untreated or treated with cycloheximide (CHX, 5 µg/ml) for 16 h. (a, b) MHC class I protein expression was measured by FACS. The percentage of positive cells (a) and the mean of fluorescence intensity (b) were quantified. Results are means ± SEM of 4 independent experiments. (c, d)
EndoC-βH1 cells were treated with cycloheximide (CHX, 5 μg/ml) for 24 h or 48 h. Protein expression was measured by western blot and representative images of 3-4 independent experiments are shown (c). Densitometry results are shown for β-actin (black squares), β-catenin (black circles) and BIP (open circles) (d). Values were normalised by the value at time 0 of each protein considered as 1. *p<0.05, **p<0.01 and ***p<0.001 vs “no CHX” and the same pre-treatment (a, b) (paired t test) or vs time 0 (d) (one way ANOVA).

Supplementary reference list