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Absence of IFN-β impairs antigen presentation capacity of splenic dendritic cells *via* down-regulation of Hsp70[1]

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Abstract

Type I interferons (IFNs) play a key role in linking the innate and adaptive arms of the immune system. Although produced rapidly in response to pathogens, IFNs are also produced at low levels in the absence of infection. In the present study we demonstrate that constitutively produced IFNs are necessary *in vivo* to maintain dendritic cells (DCs) in an “antigen presentation competent” state. Conventional dendritic cells (cDCs) isolated from spleens of IFN-β or IFNs receptor (IFNAR) deficient mice exhibit a highly impaired ability to present antigen and activate naive T cells. Microarray analysis of mRNA isolated from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs revealed diminished expression of two genes that encoded members of the heat shock protein 70 (Hsp70) family. Consistent with this observation, pharmacological inhibition of Hsp70 in cDCs from wild type (WT) mice impaired their T cell stimulatory capacity. Similarly, the antigen presentation ability of splenic cDCs isolated from Hsp70.1/3$^{-/-}$ mice was also severely impaired in comparison to WT cDCs. Thus, constitutive IFN-β expression regulates Hsp70 levels in order to help maintain DCs in a competent state for efficient priming of effector T cells *in vivo*.

Keywords

Dendritic cells; T cells; MHC; Antigen Presentation/Processing; Tolerance

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Introduction

Dendritic cells (DC)[2] are essential for the induction of specific immune responses and represent the most important cellular link between the innate and the adaptive immune system (1-5). DCs are found in most tissues where they capture and transport antigen to draining lymph nodes. During this migration, DCs mature and become highly stimulatory for T as well as B cells (6). In addition to DCs that emigrate from peripheral tissues, resident DCs can also be found in lymphoid organs such as spleen. These DCs are crucial for the sampling of blood borne antigens or pathogens (2).

DCs are generally considered as "professional antigen presenting cells", in which two principal antigen presentation pathways can be distinguished. Endogenous antigens like self- or viral components are presented *via* MHC class I (MHC I) molecules to $CD8^+$ T cells, while exogenous antigens are presented *via* MHC class II (MHC II) to $CD4^+$ T cells. In addition, DCs have the unique capacity to deliver exogenous antigens into the MHC I presentation pathway – a process known as cross presentation. This enables $CD8^+$ T cells to respond against antigens that are not directly expressed within DCs (7-9). The development, migration, maturation and function of DCs are critically influenced by cytokines produced in their surroundings (10,11), including type I interferons (IFNs).

IFNs encompass a large family of closely related cytokines comprising at least 13 IFN-α isotypes and a single IFN-β. Both IFN-α and IFN-β exert their activity through a common receptor IFNAR (12). IFN-β is thought to be the master regulator in that it is rapidly induced and can, in turn, induce the other IFNs isotypes (13,14). Furthermore, even in the absence of infection spontaneous IFN-β production, albeit at low level, is known to occur (12,15,16). These spontaneously produced IFNs contribute to host defense and cell growth in a manner similar to those induced by pathogens. In addition, constitutive production of IFNs is crucial for maintaining cells in a "primed" state and thus enabling them to mount a rapid and robust response upon encounter of external stimuli. It has thus been proposed that the absence or dysregulation of the basal constitutive IFNs signaling could be the reason for development of certain diseases (12,15-18).

In the present study, we addressed the question whether spontaneously produced IFNs play a role in the development of cell mediated immunity. Comparing the function of splenic conventional DCs (cDCs) from WT mice and mice deficient in either IFN-β or IFNAR, we found that IFN-β serves as a crucial factor for maturation of the T cell stimulatory capacity of cDCs *via* MHC I and MHC II. In its absence, we detected a lower number of specific MHC/peptide complexes at the surface of splenic cDCs. We also found that the diminished T cell stimulatory capacity of splenic cDCs occasioned by the *in vivo* absence of IFN-β is due to low expression of heat shock protein 70 (Hsp70), which is required for efficient generation of stable MHC/peptide complexes expressed on the cell surface of cDCs. Consistent with these findings, cDCs from Hsp70 deficient mice ($Hsp70.1/3^{-/-}$) were impaired in their capacity to present soluble antigens to naive T cells.

Materials and Methods

Mice

Female $IFN\text{-}\beta^{-/-}$(14), $IFN\text{-}\beta^{+/+}$, $IFNAR^{-/-}$ (19) C57BL/6 mice, OT I and OT II mice (20,21) were bred at the animal facility of the Helmholtz Centre for Infection Research (HZI). Female

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[2]**Abbreviations used in this paper**: **DC**, dendritic cells; **IFNs,** type I interferons; **IFNAR,** type I IFNs receptor; **cDCs,** conventional dendritic cells; **Hsp,** heat shock protein; **rIFN-β,** recombinant murine IFN-β; **poly I:C,** polyinosinic-polycytidylic acid; **DSG,** 15-deoxyspergualin;

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C57BL/6 mice were obtained from the Harlan-Winkelmann (Borchem, Germany and AN Venvay, The Netherlands). The initial generation of Hsp70.1/3 knockout mice has been previously described (22). The C57BL/6 Hsp70.1/3$^{-/-}$ mice were derived by transfer from a 129 background into the C57BL/6 background and were raised at Washington University School of Medicine in St. Louis. All mice were used between 8 to 12 weeks of age. Mice were bred and maintained in specific pathogen free conditions. Mouse care and experimental procedures were performed under approval of local authority LAVES.

Cell lines

The B3Z T cell hybridoma (23) specific for the H-2·K^b/SIINFEKL complex was maintained in IMDM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The hybridoma 25-D1.16 secreting an IgG1κ mAb specific for the pOV8·H-2Kb (24) was kindly provided by Dr Ronald Germain (NIAID). Antibody was purified and conjugated with FITC according to standard procedures.

Isolation of splenocytes

Spleen cells were prepared by gentle flushing out the splenocytes with IMDM supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) and 10% FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine. Erythrocytes were lysed for 2 minutes in ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and washed two or three times in PBS. Cell clumps were removed by passage through a 50 μm nylon filter. Splenocyte preparation was carried out strictly on ice. Splenic cells were than stained with appropriate antibodies and conventional dendritic cells were sorted (see below).

Flow cytometric analysis and cell sorting

Single cell suspensions were treated with anti-mouse CD16/CD32 BD Fc Block (2.4G2, Becton Dickinson, NJ, USA) for 10 min followed by staining with appropriate mAbs for 20 minutes on ice. Abs used in this work included CD11c (clone N418) conjugated with alophycocyanin (APC) or phycoerithrin-Cy7 (PE-Cy7), CD11b (M1/70) PE-Cy7, CD8α (53-6.7) PacificBlue, fluoresecein isothiocyanate (FITC) or phycoerythrin (PE), B220 (RA3-6B2) APC-Alexa Fluor 750, CD4 (GK1.5) PE, CD86 (GL1) FITC, CD40 (HM40-3) FITC, CD80 (16-10A1) PE, CD1d (1B1) PE, ICAM-1 (YN1/1.7.4) PE, B7-H1 (MIH5) PE, B7-H4 (MIH29) PE, B7-RP1 (HK5.3) PE (all purchased from eBioscience, San Diego, USA). H-2K^b (Y-3) FITC, I-A^b (M5/114.15.2) FITC. Anti H-2K^b/SIINFEKL complexes antibody (clone 25-D1.16) FITC was purified and conjugated with FITC in our laboratory. cDCs were sorted as cells exhibiting low side scatter (SSC^{lo}) in addition of being CD11c^{hi}CD8α$^+$CD11b$^-$, CD11c^{hi}CD8α$^-$CD11b$^+$. Flow cytometric analysis and sorting was performed using FACSCanto, LSRII and FACSAria (Becton Dickinson, NJ, USA), respectively. Final purity of cDCs was always >97%. All samples during the sorting procedure were kept at 4°C. The data were analyzed using FACSDiva (Becton Dickinson) software.

Preparation of T cells

OT I (OVA specific CD8$^+$ T cells) and OT II (OVA specific CD4$^+$ T cells) were isolated from lymph nodes (subcutaneous and mesenteric) and spleen. Single cell suspensions were further purified using the CD8 or CD4 negative isolation kits (Dynal, Oslo, Norway) containing mAb against B220, CD11b, Ter-119, CD16/32 and CD4 (for OT I isolation) or CD8 (for OT II isolation) following the protocol provided by the manufacturer. Cell preparations contained more than 90% of the desired cell population and were essentially free of CD11c^{hi} cells as determined by flow cytometry using Ab's specific for CD4 or CD8 and CD11c, respectively. For antigen presentation assays OT I or OT II cells were stained with 1μM CFDA(SE)

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(Molecular Probes, Oregon, USA) for 10 minutes at 37°C according to the manufacturer's protocol.

Analysis of antigen presentation *in vitro* and *ex vivo*

For the experiments using soluble OVA or peptides, individual APCs populations were plated in 96-well plates (Nunc, Denmark) at 2×10^4 cells per well with the indicated amount of soluble EndoGrade OVA (Profos, Regensburg, Germany), $OVA_{257-264}$ (SIINFEKL, Ana Spec Inc. San Jose, CA) or $OVA_{323-339}$ (kindly provided by Dr. W. Tegge, HZI) for 1h at 37°C in complete medium. The cells were further washed three times and resuspended in complete medium containing 2×10^5 CFSE labeled OT I or OT II cells. Proliferation of T cells was analyzed by flow cytometry after 1.5 (OT I peptide) or 2.5 days of culture. Cells were stained with anti-CD4 or anti-CD8 mAbs for 20 minutes, washed and resuspended in 200 μl of PBS containing Cy5-labeled 0.6 μm latex beads. Samples were analyzed until 2×10^4 beads were collected. The number of divided cells (propidium iodide^{low}, CFSE^{low}, $CD4^+$ or $CD8^+$) was determined as described (25). For *ex vivo* experiments mice were injected intravenously with 1 mg of OVA or 1 mg of OVA together with 200 μg poly I:C (Fluka, Steinheim). 24h later mice were sacrificed, APCs were sorted and incubated with OT I or OT II cells for 2.5 days. In some experiments murine recombinant IFN-β (R&D Systems, Minneapolis, USA) was added to the cultures of T cells and APCs.

Determination of antigen uptake and processing

Sorted DCs were incubated with 62.5 μg/ml of DQ-OVA (Molecular Probes, Oregon, USA) for 45 min at 37°C or on ice. DCs were then washed and analyzed by flow cytometry.

B3Z colorimetric assay

Sorted DCs (10^4 cells/well) were pulsed for 1 h with various concentrations of SIINFEKL peptide, washed twice and resuspended in phenol-red free RPMI (Gibco, Karlsruhe, Germany) containing 100 U/ml penicillin and 100 μg/ml streptomycin, 1% FCS and 2mM L-Glutamine. DCs were then co-cultured in a 96-well U bottomed plate with 5×10^4 B3Z cells/well overnight at 37°C. The next day 150 μl of supernatant was taken from each well and replaced with 150 μl of PBS containing 5 mM ONPG (Sigma, St. Louis, USA) and 0.5% IGEPAL-20 (Sigma, St. Louis, USA). The plate was than incubated at 37°C for 2h and optical density was measured at 450 nm with wavelength correction set at 650 nm.

Microarray Studies

RNA isolation, cDNA preparation andDNA microarray analysis of gene expression was performed at the gene array facility of the HZI. Fluorescent images of hybridized microarrays (Affymetrix, MOE-430 version 2.0) were obtained using an Affymetrix Genechip Scanner. Microarray data were analyzed using BioConductor Suite 2.1 software. All samples were repeated two times with individually sorted cells and averaged. Data discussed here have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE12392.

Quantitative real-time PCR

Total RNA was extracted from sorted APCs using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA contamination in the total RNA preparation was eliminated using DNase I (Qiagen, Hilden, Germany). Oligo$(dT)_{18}$ primers and RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Ontario) were used for reverse transcription of purified RNA. All gene transcripts were quantified by quantitative PCR with Power SYBR Green qPCR Master Mix (Applied Biosystems, Foster City, USA) and Light

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Cycler apparatus (ABI PRISM Cycler Applied Biosystems, Foster City, USA). Primers specific for Hsp70.1 were synthesized as described before (26).

Intracellular staining of Hsp70

Splenic cDCs were sorted out and stained intracellularly using Cytofix/Cytoperm kit (Becton Dickinson, NJ, USA) according to manufacturer's protocol with anti-Hsp70 (C92F3A-5) PE conjugated Ab (Stressgen, Canada).

Inhibition of Hsp70 by 15-deoxyspergualin (DSG)

DSG was a generous gift of Nippon Kayaku Co. Ltd. (Tokyo, Japan). Animals were injected intraperitoneally daily with 10 mg/kg of DSG or PBS for 6 days before splenic cDCs were sorted out and tested for their antigen presentation capacity with OT I or OT II cells in CFSE dilution assay.

Results

Splenic dendritic cells from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice are impaired in T cell stimulation

IFNs are known to be constitutively produced at low levels under non-inflammatory conditions (12). To study the influence of IFNs on antigen presentation under physiological conditions we decided to focus on freshly isolated splenic cDCs. These cells are representative of typical non migratory DCs found *in vivo* at steady state (1-3). Analysing IFN-β and IFNAR deficient mice we detected no differences with regard to percentage of various splenic cDCs subpopulations in mice with and without either IFN-β or IFNAR (Fig. 1A). Furthermore, we determined the overall number of leukocytes in several lymph nodes and spleen. Consistently, there was no significant difference observed in comparison to WT mice (Fig. 1B).

Thus, we analyzed the ability of cDCs exhibiting the markers CD11c^{hi}, CD11b$^{+/-}$, CD8α$^{+/-}$, B220^{-} from spleens of WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice to present ovalbumin (OVA) protein to CFSE labeled OT I or OT II T cells. When compared to WT, cDCs from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were severely impaired in their ability to activate such CD8^{+} and CD4^{+} T cells (Fig. 2A and 2C).

To test whether the anomaly associated with IFN-β or IFNAR deficiency affects also the presentation of preprocessed antigen, i.e. peptides, cDCs from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were loaded with MHC class I or MHC class II specific peptides (OVA$_{257\text{-}264}$ (SIINFEKL) and OVA$_{323\text{-}339}$, respectively) and then incubated with CFSE labeled OT I and OT II T cells. As shown in Fig. 2B and 2D, T cell stimulation was also highly impaired when peptide loaded cDCs from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were used.

Throughout most of the experiments we used bulk sorted splenic cDCs because during *in vitro* co-cultures with T cells the two distinct populations - CD8α^{+} DCs and CD8α^{-} DCs (myeloid DCs) from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were similarly impaired in their T cell stimulatory capacity compared to WT DCs (Fig. S1).

Deficiency in IFNs does not impair survival of cDCs *in vitro*

As IFNs provide cellular survival signals under certain conditions (27,28), we first wanted to test whether the reduced ability to stimulate T cells might be due to lower survival of cDCs from IFN-$\beta^{-/-}$or IFNAR$^{-/-}$ mice during the *in vitro* T cell stimulation assay. Splenic cDCs sensitized with OVA protein were incubated with OT I or OT II cells. After 16 and 32 hours the percentage of live cDCs was assessed by propidium iodide (PI) exclusion. WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs were equally viable under these conditions (Fig. S2A, S2B). Thus, the reduced ability to stimulate T cells *in vitro* was not due to lower survival of IFN-$\beta^{-/-}$ cDCs.

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Impaired stimulatory capacity of cDCs can be restored by supplementation with recombinant IFN-β *in vitro* or induction of IFNs with poly I:C *in vivo*

Next, we asked whether exogenous administration of IFNs could restore the impaired T cell stimulatory capacity of IFN-β$^{-/-}$ cDCs *in vitro*. Titration of murine recombinant IFN-β (rIFN-β) into co-cultures of IFN-β$^{-/-}$ cDCs and T cells showed that low amounts (0.1 U/ml) could completely restore the impaired T cell stimulatory function (Fig. 3A). However, probably due to activation of negative feedback mechanisms, addition of higher concentrations of rIFN-β (5-500 U/ml) to the co-cultures failed to restore T cell stimulatory ability of cDCs (Fig. 3A and data not shown). These results support the argument that the low levels of IFN-β produced at steady state are well optimized for maintaining of cDCs in antigen presentation competent state.

Nevertheless, in such a situation it is difficult to exclude that exogenous rIFN-β influenced T cell proliferation. A direct effect of IFNs on T cells has been well documented (28,29) although it only partially could explain our results. We could show that IFNAR$^{-/-}$ cDCs which are able to produce IFN-β (data not shown), are still inefficient in activating a T cell response (Fig. 2). In addition, when such co-cultures are complemented with recombinant IFN-β the inefficiency of T cell activation remained (Fig. 3B). This clearly demonstrates that steady state production of IFN-β is required for maintenance of proper cDC function.

Furthermore, we tested whether triggering IFNs-α *in vivo* could compensate the impaired development of T cell stimulatory capacity of cDCs from IFN-β$^{-/-}$ mice. WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice were intravenously injected with OVA alone or OVA together with polyinosinic-polycytidylic acid (poly I:C). After 24h, splenic cDCs were sorted and tested for their ability to activate the proliferation of OT I or OT II T cells. Data depicted in Fig. 3C show that IFNs-α induction by poly I:C compensated for the lack of IFN-β during cDC development *in vivo* and partially recovered their function. As expected, *in vivo* administration of poly I:C did not improve the stimulatory function of splenic cDCs from IFNAR$^{-/-}$ mice as they are completely unresponsive to IFNs signaling (Fig. 3D).

We also tested the T cell stimulatory capacity of DCs differentiated *in vitro* by incubating bone marrow cells with IL-4 and GM-CSF (BMDCs). After 8 days of culture we obtained around 80% CD11c positive cells from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice. Here, WT and IFN-β$^{-/-}$ or IFNAR$^{-/-}$ BMDCs were comparable in their ability to stimulate the proliferation of T cells (data not shown). This suggests that the influence of IFNs observed *ex vivo* is greatly dependent on the overall stimulatory context under which the DCs develop.

IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs display normal antigen capture and processing

A differential ability to acquire and process soluble antigen could account for the diminished stimulatory capacity of cDCs in the absence of the IFNs system. Therefore, the efficiency of splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice to take up and degrade soluble OVA was assessed. We used DQ-OVA, which generates fluorescent byproducts upon degradation. As shown in Fig. 4, splenic cDCs from all groups acquired and generated comparable amounts of fluorescent DQ-OVA products. This was true for different DQ-OVA concentrations tested (data not shown). Thus, changes in antigen uptake and degradation could not account for the impaired T cell stimulatory capacity of cDCs from IFN-β$^{-/-}$ or IFNAR$^{-/-}$ mice.

Similar expression of MHC and co-stimulatory molecules on cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice

The maturation status of DCs is known to be a fundamental factor for proper T cell stimulation. One of the mechanisms by which constitutive IFNs signaling could influence T cell stimulation is to enhance expression of MHC or adhesion and co-stimulatory molecules on the surface of

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DCs (18). However, analysis of splenic CD8α$^+$ and CD8α$^-$ cDCs for MHC I and MHC II as well as co-stimulatory or adhesion molecules like CD86, CD80, CD40 and ICAM-1 indicated no significant differences between WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice in both cDC populations (Fig. 5, Fig. S3). Therefore, the impaired function of splenic cDCs from IFN-β and IFNAR deficient mice was not due to lower expression of such surface molecules.

An intact IFNs system is required for the efficient formation of stable MHC/peptide complexes at the surface of splenic cDCs

The fact that an impaired T cell stimulatory capacity of cDCs from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice was also found for the presentation of peptides, not requiring further processing, as well as unimpaired DQ-OVA degradation suggested that the phenotype of IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs was most likely due to a defect in peptide presentation rather than antigen processing steps. We therefore decided to study the MHC/peptide complexes on the surface of cDCs. We employed B3Z cells, a H-2·K^b restricted T cell hybridoma specific for the OVA epitope SIINFEKL (OVA$_{257-264}$), which upon T cell receptor (TCR) activation expresses β-galactosidase (23). The activation of B3Z cells, being a hybridoma, is independent of co-stimulation (30) thus, their activation should only be dependent on the concentration of MHC I/peptide complexes recognizable by the TCR. Therefore, splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice were loaded with OVA$_{257-264}$ peptide and tested for their ability to activate B3Z cells. As shown in Fig. 6A and 6B, cDCs from mice deficient in IFN-β or IFNAR exhibited lower stimulatory capacity. These results confirmed that the impaired function of splenic cDCs from such mice is not due to lower levels of co-stimulatory molecules, but strongly suggested that the defect was in the process of MHC I/peptide complex formation. By using the 25-D1.16 antibody which recognizes SIINFEKL bound to the H-2·K^b molecule (24), we confirmed our interpretation. DCs from either IFN-β$^{-/-}$ or IFNAR$^{-/-}$ mice had lower levels of MHC I/SIINFEKL complexes compared to cDCs from WT mice (Fig. 6C).

Deficiencies in the IFNs system leads to decreased expression of Hsp70 in splenic cDCs

To understand the molecular basis for the decreased formation of MHC/petide complexes, splenic DCs RNA from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice was analyzed by microarrays for expression of genes known to be involved in antigen processing and presentation, co-stimulation or IFNs response. Extensive analysis of the microarrays indicated that most of these genes were unaltered in cDCs from the knockout mice (Fig. S4A-C). The only dramatic difference found was in the expression of the heat shock protein Hsp70.1 and Hsp70.3 genes. The expression of these two genes was significantly lower in cDCs from IFN-β$^{-/-}$ (approximately 15-20 fold down-regulated in comparison to WT) and IFNAR$^{-/-}$ (approximately 75-150 fold down-regulated in comparison to WT) mice (Fig. 7A).

To verify the above findings, we first stained for intracellular Hsp70 protein. As shown in Fig. S5 levels of Hsp70 were indeed lower in IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs compared to WT cDCs. Most likely due to presence of other highly homologous members of the Hsp70 family and the low sensitivity of the antibody, differences were not very pronounced. However, in confirmation of the microarray data, transcriptional levels of Hsp70.1 were severely decreased in DCs from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice in comparison to WT (Fig. 7B). Moreover, treatment with low amounts of rIFN-β (0.1 U/ml) increased Hsp70 levels in both WT and IFN-β$^{-/-}$ DCs, whereas treatment with 5 U/ml of rIFN-β did not markedly change the Hsp70 levels (Fig. 7C). This correlates well with the functional restoration of IFN-β$^{-/-}$ cDCs at low, but not at high concentrations of rIFN-β. Consistent with this finding, 24h of poly I:C administration up-regulated Hsp70.1 levels in WT as well as in IFN-β$^{-/-}$ cDCs (Fig. 7D).

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Inhibition of Hsp70 by 15-deoxyspergualin leads to impairment of antigen presentation

To test for a possible causative link between Hsp70 expression and antigen presentation, we used 15-deoxyspergualin (DSG), a pharmacological inhibitor of Hsp70. DSG is a synthetic derivative of spergualin from *Bacillus laterosporus* and binds to Hsp70 and Hsp90 (31-33). Therefore, we treated mice for 6 days with DSG and then tested the splenic cDCs of such mice for Hsp70 expression. Intracellular staining revealed that DSG treatment led to partial reduction of Hsp70 level in WT cDCs (Fig. 8A). In contrast, expression of surface molecules involved in T cell stimulation was not affected by this treatment (Fig. S6).

We then tested cDCs isolated from DSG treated mice for their capacity to stimulate $CD4^+$ and $CD8^+$ T cells. Clearly, cDCs from DSG treated mice exhibited a reduced ability to stimulate OT I or OT II T cells compared to cDCs from untreated mice, independent of whether protein or peptides were used as an antigen (Fig. 8B). This was consistent with the claims that DSG abrogates the ability to present antigen in the context of both MHC I and MHC II (31-34).

In addition, compared to untreated control, cDCs from DSG treated mice revealed lower surface levels of MHC/peptide complexes (Fig. 8C) suggesting that Hsp70 is necessary for efficient formation of MHC/peptide complexes. Thus, by supporting the expression of Hsp70, constitutive IFN-β expression *in vivo* helps to maintain cDCs in a primed and competent state for antigen presentation.

Splenic dendritic cells from Hsp70.1/3$^{-/-}$ mice are impaired in T cell stimulation

In order to explicitly demonstrate the involvement of the Hsp70.1 and Hsp70.3 proteins in antigen presentation and thus confirm that in the absence of IFN-β or IFNs signaling down-regulation of Hsp70 result in impaired antigen presentation we utilized Hsp70.1/3 double knockout mice (22). The surface phenotype of cDCs from Hsp70.1/3 $^{-/-}$ mice appeared to be very similar to the surface phenotype of cDCs from WT (data not shown). To test the antigen presentation capacity of Hsp70.1/3 $^{-/-}$ cDCs, cells were sorted and loaded with appropriate OVA peptides or whole protein and incubated with OT I or OT II transgenic T cells. The results clearly show that cDCs from Hsp70.1/3$^{-/-}$ are impaired in their ability to present OVA derived peptides as well as whole protein to naive T cells when compared to WT cDCs (Fig. 8D). This further substantiates our finding that down-regulation of Hsp70 in the absence of IFN-β or IFNs can alter antigen presentation. The discovery that IFNs signaling regulates MHC/peptide complex formation by Hsp70 proteins highlights a hitherto unrecognized mechanism *via* which IFNs might regulate presentation of self antigens in the steady state and has, therefore, important consequences for our understanding of how regular homeostatic conditions are maintained in the immune system.

Discussion

IFNs, found in high amounts in cells exposed to viruses, were first characterized and named as such on the basis of their antiviral activity. It is now well known that IFNs have widely overlapping, pleiotropic and immunomodulatory effects and their production is not the sole preserve of viral infections but they are also induced in response to bacterial and parasitic infections (12,15,16). IFNs represent important immunomodulators for the innate as well as the adaptive arm of the immune system (16,18). They exert broad regulatory effects and various subtypes of DCs are affected by these cytokines (12,15-18). For instance, IFN-α can promote antigen cross-presentation by enhancing endosomal processing, up-regulating the expression of co-stimulatory molecules and augmenting dendritic cell viability in settings of viral infection (18,27,29,35-37). Additionally, direct stimulation of T cells by IFN-α has been shown to be essential for efficient induction of cross-priming (29). Furthermore, IFN-α/β were described as crucial survival factors for activated T cells (28). Importantly apart from that, even in the

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absence of infection spontaneous low level production of IFN-β has been shown to occur (12).

The host response elicited by IFNs is largely dependent on signal strength. Most of the studies carried out to date have focused on the cellular effects induced by the high levels of IFNs elicited under inflammatory conditions. However, whether the low levels of IFNs produced under non-inflammatory conditions have an important housekeeping immune function is not known. We now show that the low but constitutive production of IFN-β is necessary for maintaining DCs in a state competent for antigen presentation. Compared to those from WT mice, DCs freshly isolated from spleens of IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were found to be highly impaired in antigen presentation to $CD4^+$ and $CD8^+$ T cells. This defect could in part be rectified with exogenous rIFN-β or through *in vivo* induction of IFNs-α using synthetic dsRNA. Interestingly, restoration of function was possible with extremely low amounts of rIFN-β, probably mimicking amounts produced constitutively. Failure of IFN-$\beta^{-/-}$ DCs cultured with high rIFN-β levels to activate T cell proliferation might be attributed to negative feedback mechanisms activated by a strong IFN-β signal.

The function of DCs is not only influenced by cytokines present in their environment, but also by other cells of the immune system, particularly T cells. Therefore, splenic cDCs isolated from IFN-β deficient mice theoretically could be altered due to an effect of IFN-β deficiency on T cells. It was shown before that IFN-α and IFN-β have direct effect on activated T cells and prevent their death during inflammatory conditions (28). Activated T cells can provide feedback signals to DCs and induce their maturation. This can be mediated by cytokines produced by T cells as well as by cell-cell interactions, including CD40L-CD40 interactions (38). However, this phenomenon is unlikely to play a significant role in steady state conditions because of the lack of activated T cells. T cell mediated conditioning of DCs *via* B7-H1 in homeostasis has recently been shown to play an important role in inducing DC maturation (39). However, as surface expression of B7-H1 was not altered on IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs in comparison to WT cDCs, one may assume that this is not the case in our situation. Moreover, we demonstrated that rIFN-β treatment *in vitro* can regulate cDCs function and Hsp70 levels.

Potential roles of IFNs in antigen presentation have previously been postulated. However, such effects have only been observed for high levels of IFNs reminiscent of inflammation. For instance, induction of IFNs in DCs by dsRNA and LPS *in vitro* was shown to up-regulate costimulatory and MHC molecules hence enhancing their ability to activate $CD8^+$ T cells (17). Conversely, our results show that the antigen presentation defects in splenic IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ DCs are neither due to low expression of co-stimulatory and MHC molecules nor a block in antigen capture and processing. We demonstrate that the defect is due to a blockade in a step downstream of antigen processing: MHC/peptide complex formation. Furthermore, the data presented here strongly suggest that impairment in the MHC/peptide complex formation step caused by the absence of IFNs signaling is due to down-regulation of Hsp70.

The 70 kD Hsps Hsp70.1 and Hsp70.3 belong to a larger, highly homologous and conserved gene family whose expression can be significantly induced in response to a number of pathophysiological conditions including pathogen exposure (40). In addition to a generalized role in protein folding and transport, the proteins also have distinct functions in the promotion of antigen processing and presentation (31,41-43). Hsp70 proteins are involved in chaperoning proteins/peptides during degradation and during antigen presentation *via* MHC I as well as *via* MHC II. Hsp70 has been shown to physically associate with the transporter associated with antigen processing (TAP) hence enabling efficient loading of chaperoned peptides onto MHC I molecules (31,41,44). In addition, a role for Hsp70 in antigen presentation by MHC II molecules has also been described (31,45).

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By microarray and quantitative real-time PCR (qRT-PCR) analysis we found that in the absence of a functional IFNs system *in vivo*, the expression of Hsp70.1 and Hsp.70.3 in splenic cDCs was significantly down-regulated. In addition, partial blockade of Hsp70 protein in WT cDCs using the pharmacological inhibitor DSG resulted in a diminished ability to activate T cells. This correlated with a decrease in the surface MHC/peptide complexes on WT splenic DCs after treatment with DSG. These results were further substantiated when we analyzed the ability of cDCs from Hsp70.1/3$^{-/-}$ mice to present soluble OVA to naive CD4^{+} and CD8^{+} T cells. The function of such cDCs was highly impaired when compared to WT cDCs, suggesting that indeed altered levels of Hsp70 in IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs are responsible for impaired antigen presentation in the steady state.

From data presented here, one could expect that in the absence of chaperones, like Hsp70, when formation of antigenic MHC/peptide complexes is impaired, total levels of surface MHC molecules should be diminished. Nevertheless, our experiments show no differences between WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs in total surface MHC I and MHC II expression. Likewise, the analysis of the surface phenotype of Hsp70.1/3$^{-/-}$ cDCs showed no significant difference in comparison to WT cDCs. Possibly, Hsp70 might be required for efficient presentation of particular peptides, like the ones employed in our study, but not for others. Therefore, lack of Hsp70 might not reflect in changes of total surface MHC levels. The exact molecular reason for this phenomenon remains to be elucidated.

The discovery that basal IFNs production regulates efficiency of MHC/peptide complex formation *via* the expression of Hsp70 is a novel and important finding not only in the context of pathogen recognition but also in homeostasis. Not all pathogens are associated with robust IFNs production. Therefore by sustaining Hsp70 expression, constitutively produced IFNs probably ensure that DCs are kept in a primed state for efficient presentation of antigens from such pathogens. An equally or more important function could be in the maintenance of tolerance to self antigens. DCs that capture and present antigen under non-inflammatory conditions are generally believed to acquire tolerogenic properties and generate regulatory T lymphocytes that potentiate tolerogenic responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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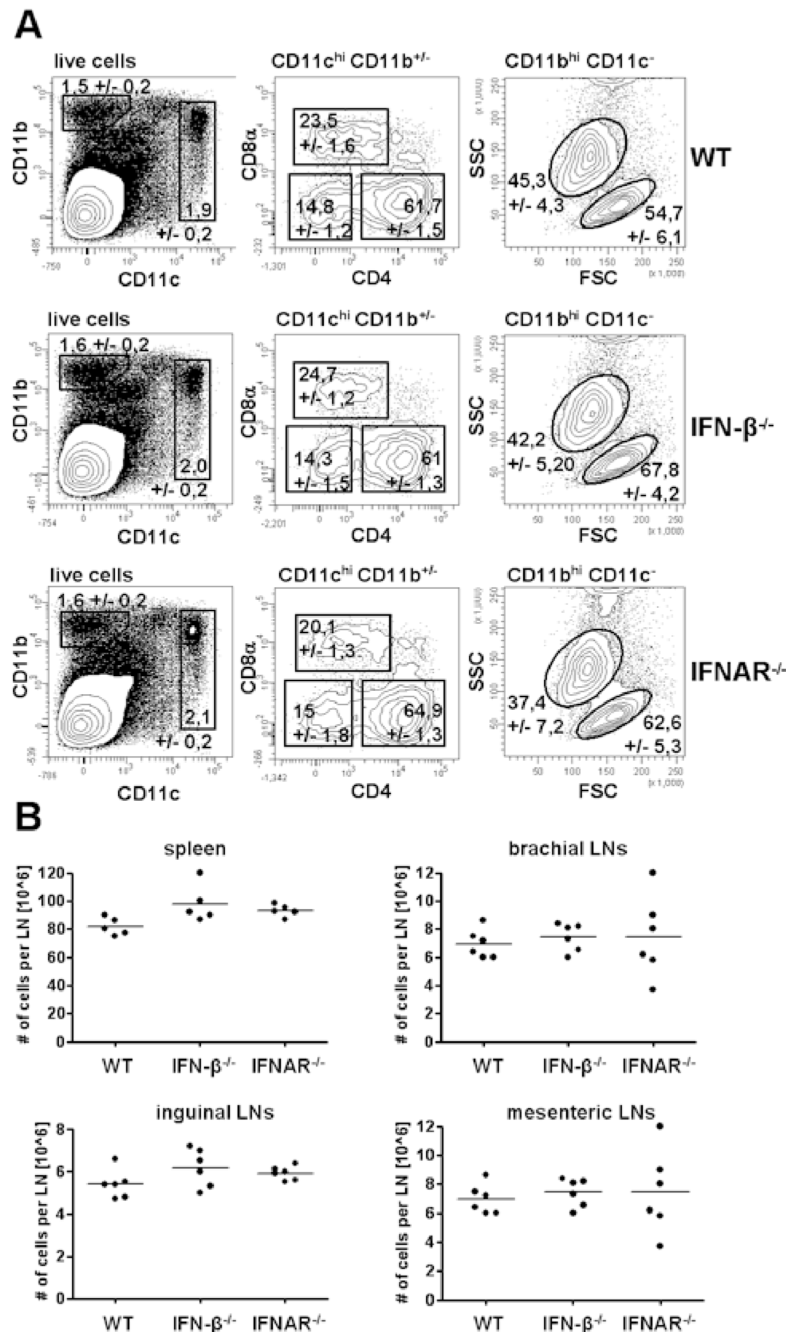

Figure 1. Similar percentage and frequency of different splenic cDCs populations, macrophages and granulocytes in spleens of WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice

(**A**) Splenocytes from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ were isolated, stained for following markers: CD11c, CD11b, CD4, CD8α and B220 and analyzed by flow cytometry. Data are representative of three independent experiments with at least 5 mice per group. (**B**) Similar number of leukocytes per lymph node or spleen in WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice.

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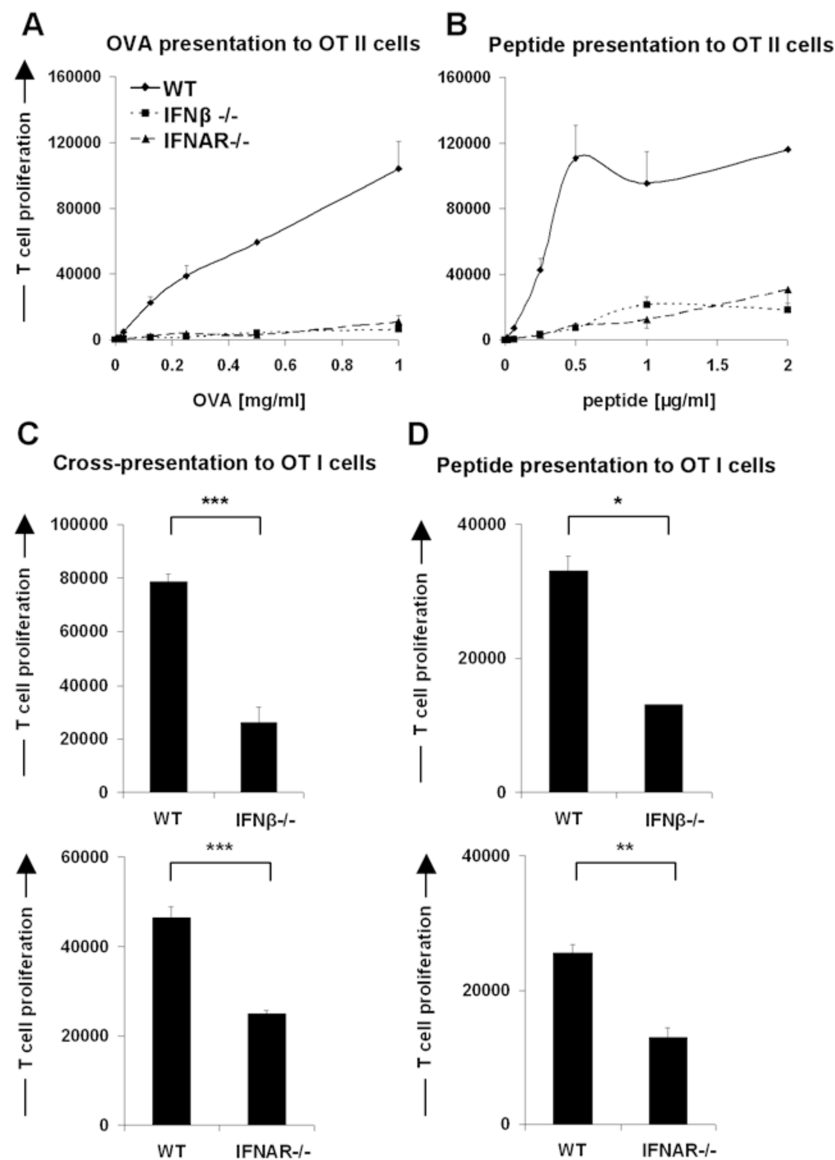

Figure 2. Splenic conventional dendritic cells (cDCs) from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice are impaired in their antigen presentation capacity of soluble OVA as well as OVA derived peptides in the context of both MHC I and MHC II

The purified OT I or OT II transgenic T cells were labeled with CFDA(SE) and incubated for 1.5 days (OT I peptide) or 2.5 days with splenic cDCs (CD11c^{high}, CD11b$^{+/-}$, CD8α$^{+/-}$, B220^{-}) in ratio 10:1. cDCs from C57BL/6 WT, IFN-β$^{-/-}$ and from IFNAR$^{-/-}$ mice were preloaded with OVA$_{257-264}$ (class I-restricted), OVA$_{323-339}$ (class II-restricted) peptides or whole OVA protein for 1h and further were washed intensively. (**A** and **B**) antigen presentation in the context of MHC II, (**C** and **D**) antigen presentation in the context of MHC I. OVA concentration 250 μg/ml, OVA$_{257-264}$ peptide concentration 10 ng/ml. The proliferative response of T cells was enumerated by flow cytometry. Data are representative of at least three mice for WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ in multiple independent experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; ** P<0.01; *** P<0.005

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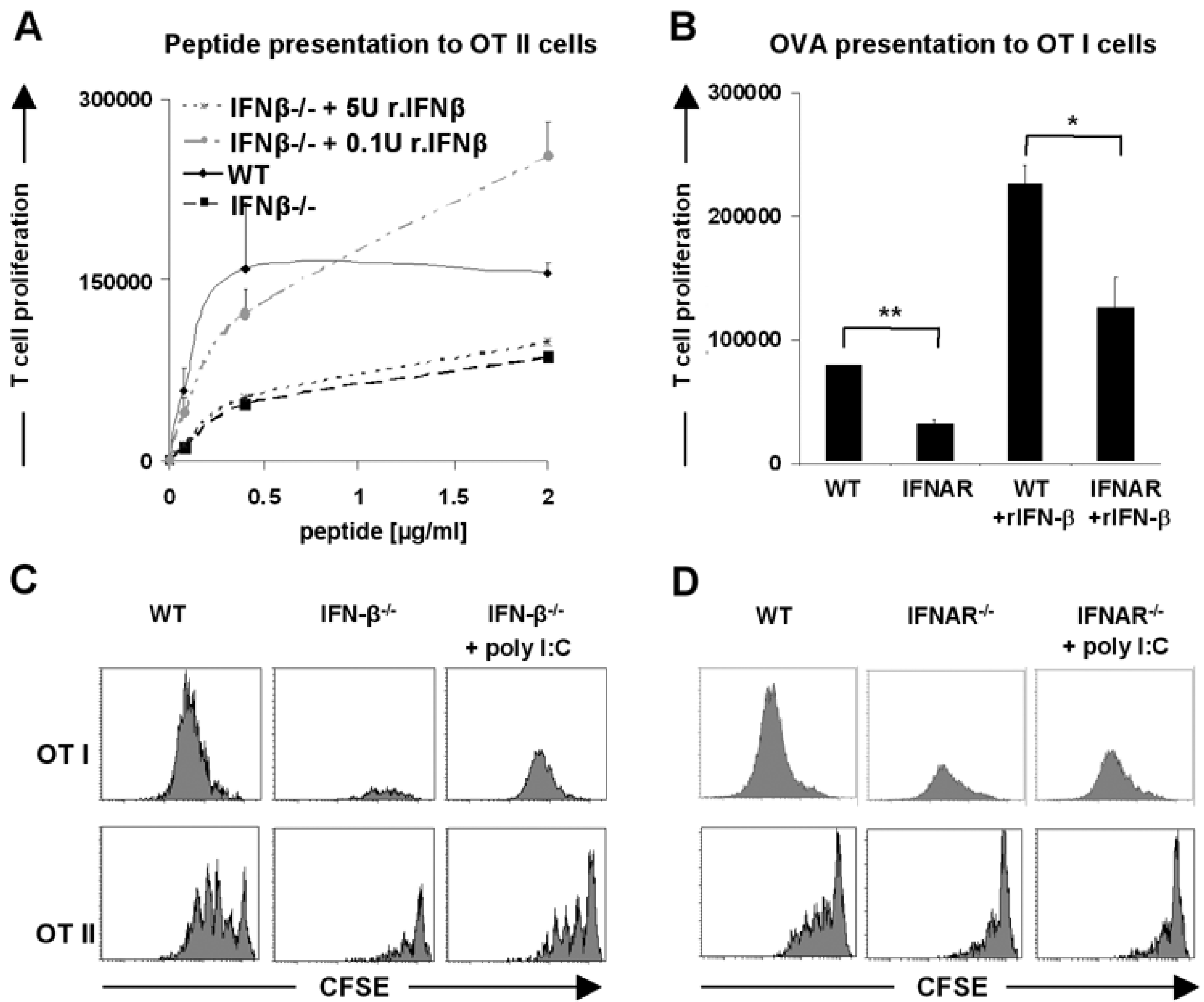

Figure 3. IFN-β is required *in vitro* during cDC-T cell contact as well as *in vivo* during cDCs development

(**A**) Addition of low amounts of murine recombinant IFN-β (0.1 U/ml) leads to efficient recovery of impaired ability to present antigen by splenic cDCs from IFN-$\beta^{-/-}$ mice. OT I and OT II cells after purification were labeled with CFDA(SE). Splenic cDCs from WT and IFN-$\beta^{-/-}$ mice were sorted out from spleens of at least three mice, loaded with indicated concentration of OVA peptide for 1h, washed intensively and cocultured with T cells for 2.5 days in presence of recombinant murine IFN-β. (**B**) Enhanced proliferation of T cells accompanying impaired function of IFNAR$^{-/-}$ cDCs in the presence of exogenously added low amounts of rIFN-β. Splenic cDCs from WT and IFNAR$^{-/-}$ mice were sorted out from spleens of at least three mice per group, loaded with 250 µg/ml of OVA protein for 1h, washed intensively and cocultured with OT I T cells for 2.5 days in presence of 0.1 U/ml of recombinant murine IFN-β. (**C** and **D**) *In vivo* induction of IFNs by poly I:C leads to partial restoration of function of splenic cDCs from IFN-$\beta^{-/-}$ mice. WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were treated with poly I:C together with OVA or OVA alone, 24h later splenic cDCs were sorted out and incubated *in vitro* with CFDA (SE) labeled OT I or OT II cells for 2.5 days. The proliferative response of T cells was enumerated by flow cytometry. Data are representative of two independent experiments.

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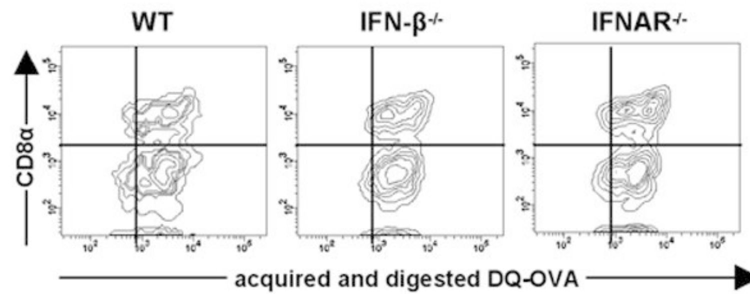

Figure 4. Lack of IFN-β and IFNs signaling has no influence on proper uptake and processing of soluble antigen by splenic cDCs

Splenic cDCs from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were sorted out and incubated for 1h with 62.5 μg/ml of DQ-OVA. Further cells were washed and BODIPY fluorescence was measured using flow cytometry. Density plots show DQ-OVA proteolysis by splenic cDCs. Data are representative of three independent experiments and of at least three mice per group.

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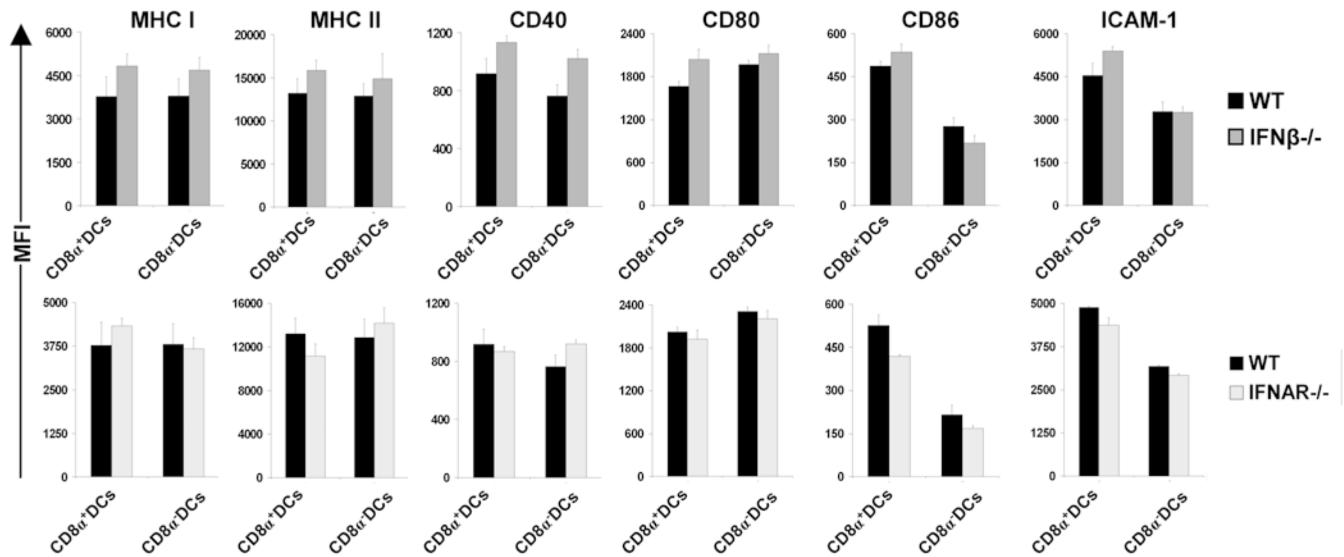

Figure 5. Similar surface phenotype of splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice

Splenocytes isolated from C57BL/6 WT, IFN-β$^{-/-}$ or IFNAR$^{-/-}$ mice were stained and gated on the basis of CD11c, CD11b, CD8α and B220 on two populations of cDCs. Graphs show MFI (Mean Fluorescence Intensity) values for expression of indicated markers. No major differences between DCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice were observed. MFI of each marker was measured for at least five mice per group. Data are representative of 5 independent experiments.

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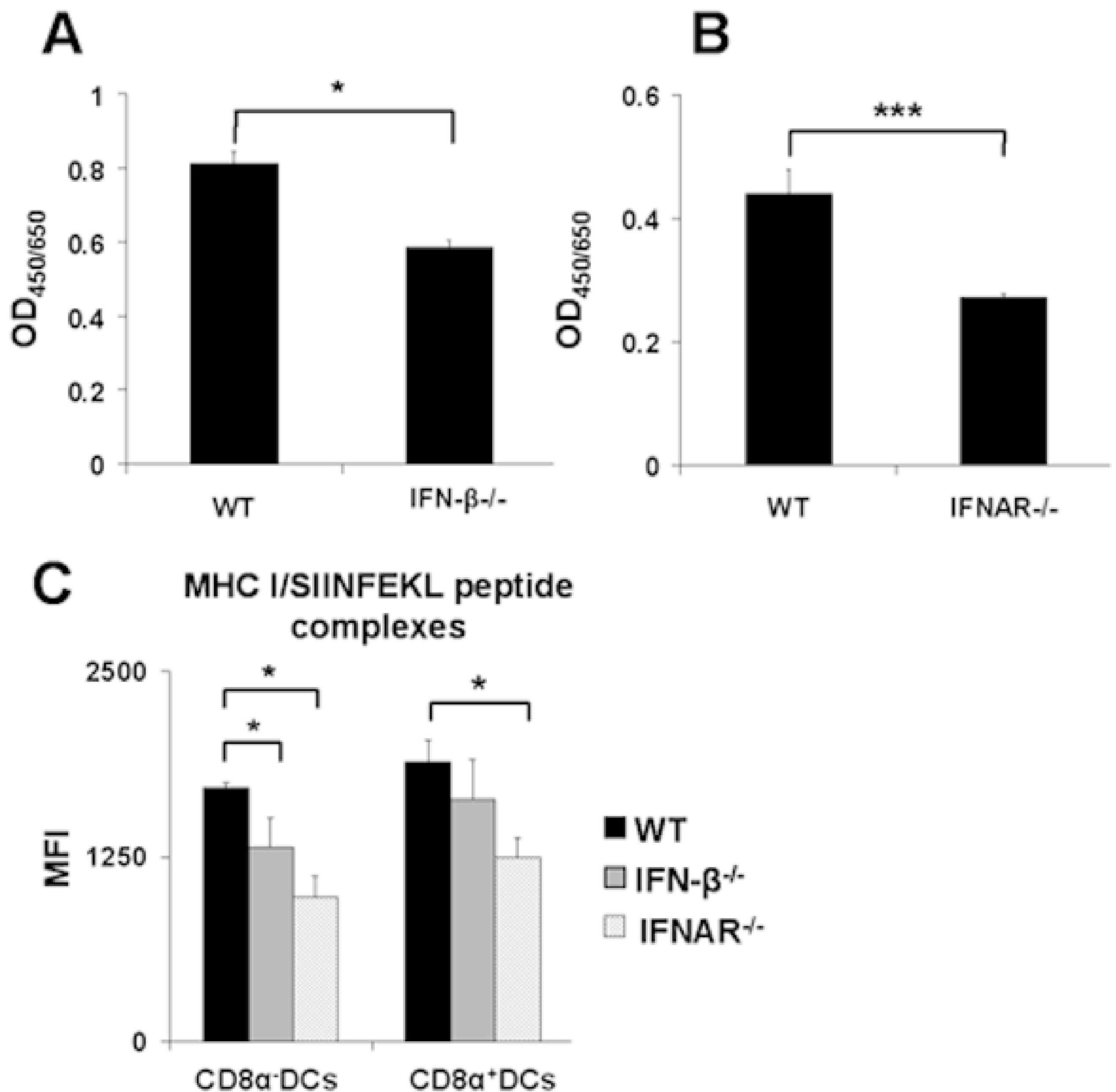

Figure 6. Splenic cDCs from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice form lower levels of MHC I/SIINFEKL complexes

Splenic cDCs were sorted out from spleens of WT, IFN-β$^{-/-}$ or IFNAR$^{-/-}$ mice, pulsed with SIINFEKL peptide ($OVA_{257-264}$) for 1h, washed intensively and (**A, B**) co-cultured with the SIINFEKL/H-2K^{b} restricted B3Z hybridoma T cells for 24h. Cells were then lysed and monitored for LacZ expression by the introduction of ONPG substrate. Optical density was measured at 450 nm with wavelength correction set at 650 nm. (**C**) cDCs were stained with 25-D1.16 antibody, MFI was measured by flow cytometry, graphs show values for 100 ng/ml of SIINFEKL peptide, cells were gated on CD8α^{+}DCs and myeloid DCs (CD8α^{-}). Results are representative of at least three mice for WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ in three independent

experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; *** P<0.005

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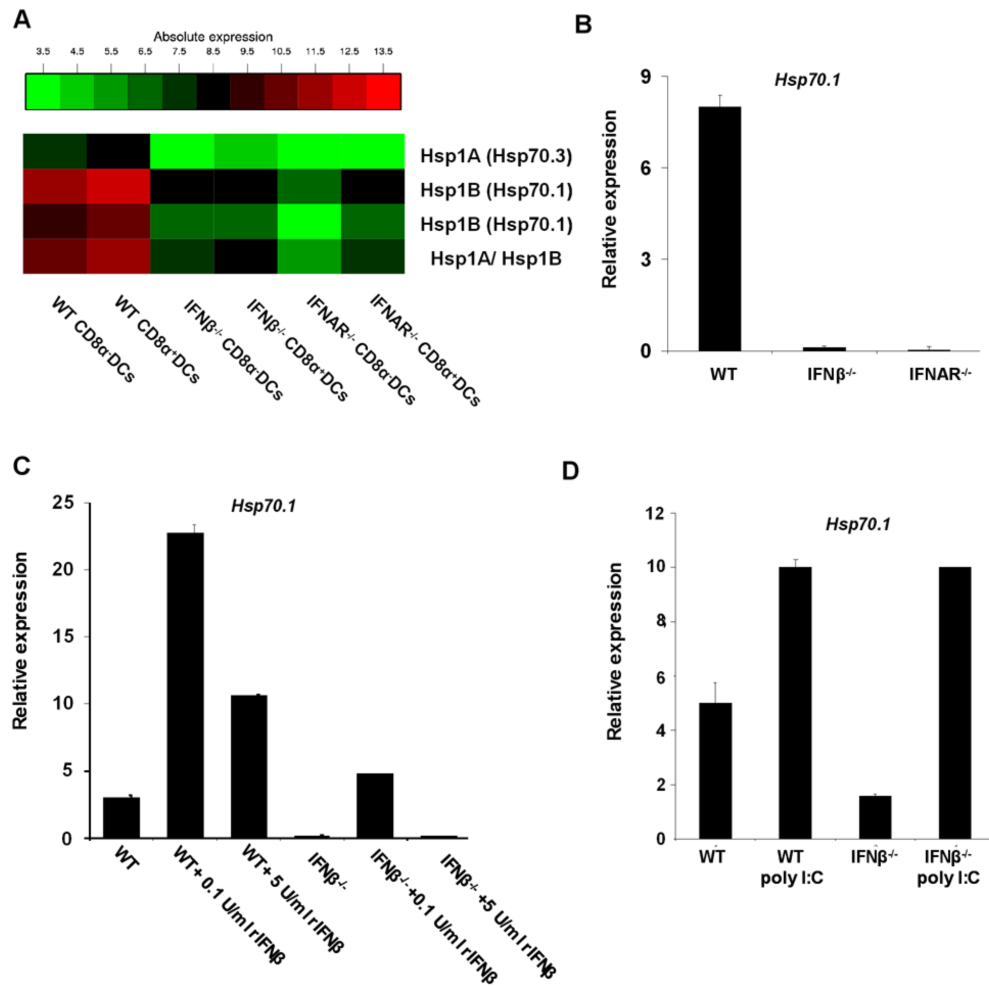

Figure 7. Microarray analysis of splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice showed down-regulation of heat shock protein 70.1 and 70.3 (Hsp70.1 and Hsp70.3) in knockout mice

(**A**) Affymetrix gene array analysis of splenic cDCs. RNA was prepared from sorted splenic CD8α^{+}DCs and myeloid DCs (CD8α^{-}) from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice. All samples were repeated twice with individually sorted cells and averaged. (**B**) Quantitative real-time PCR (qRT-PCR) of RNA from splenic cDCs sorted from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice. (**C**) qRT-PCR of RNA from splenic cDCs untreated and *in vitro* treated with 0.1 U/ml and 5 U/ml of murine recombinant IFN-β for 3h. (**D**) qRT-PCR of RNA from splenic cDCs 24h after administration of poly I:C. Results are representative of at least three samples from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice in three independent experiments.

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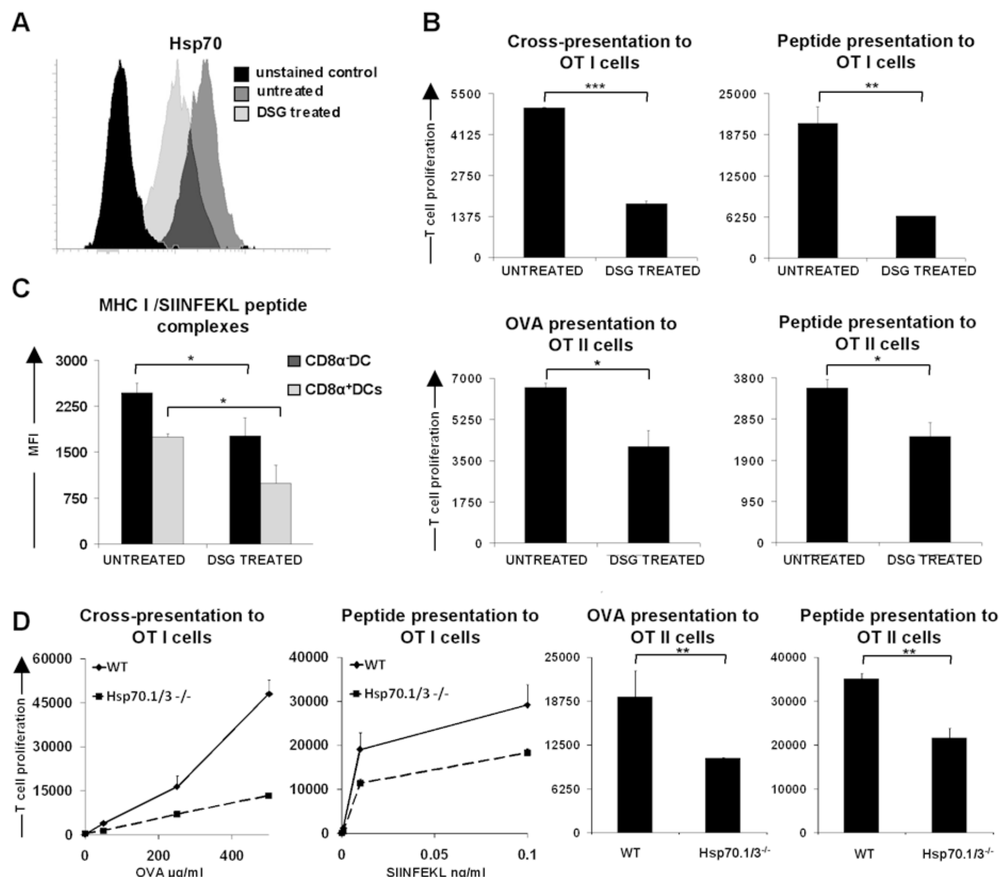

Figure 8. *In vivo* inhibition of Hsp70 by 15-deoxyspergualin or knockout of Hsp70.1 and Hsp70.3 leads to impaired antigen presentation by splenic cDCs

(**A**) Intracellular staining of Hsp70 in splenic cDC from mice untreated and treated with 15-deoxyspergualin. (**B**) Antigen presentation assay with splenic cDCs from untreated mice and mice treated with 15-deoxyspergualin. Splenic cDCs were incubated with purified, CFDA(SE) labeled OT I or OTII cells for 1.5 days (class I restricted peptide) and 2.5 days. $OVA_{257\text{-}264}$ peptide concentration 10 ng/ml, $OVA_{323\text{-}339}$ 2 µg/ml, OVA protein 1mg/ml (class II presentation) or 500 µg/ml (class I presentation). The proliferative response of T cells was enumerated by flow cytometry. Data are representative of at least five mice for WT untreated and DSG treated in three independent experiments. (**C**) DCs from untreated and DSG treated mice were loaded for 1h with 100 ng/ml of SIINFEKL peptide, washed and stained with 25-D1.16 antibody recognizing MHC I/SIINFEKL peptide complexes. Graph shows MFI values for five mice per group. Data are representative of two independent experiments. (**D**) Antigen presentation assay with splenic cDCs sorted from Hsp70.1/$3^{-/-}$ and WT mice. Cells were loaded with appropriate OVA derived peptides or whole OVA, washed and incubated *in vitro* with CFDA(SE) labeled OT I or OTII cells for 1.5 days (class I restricted peptide) and 2.5 days. $OVA_{323\text{-}339}$ peptide concentration 2 µg/ml, OVA protein 1mg/ml (MHC II presentation). The proliferative response of T cells was enumerated by flow cytometry. Data are representative of 3 mice for Hsp70.1/$3^{-/-}$ and WT in two independent experiments. Statistical significance was determined using the paired Student's t test. * $P<0.05$; ** $P<0.01$; *** $P<0.005$