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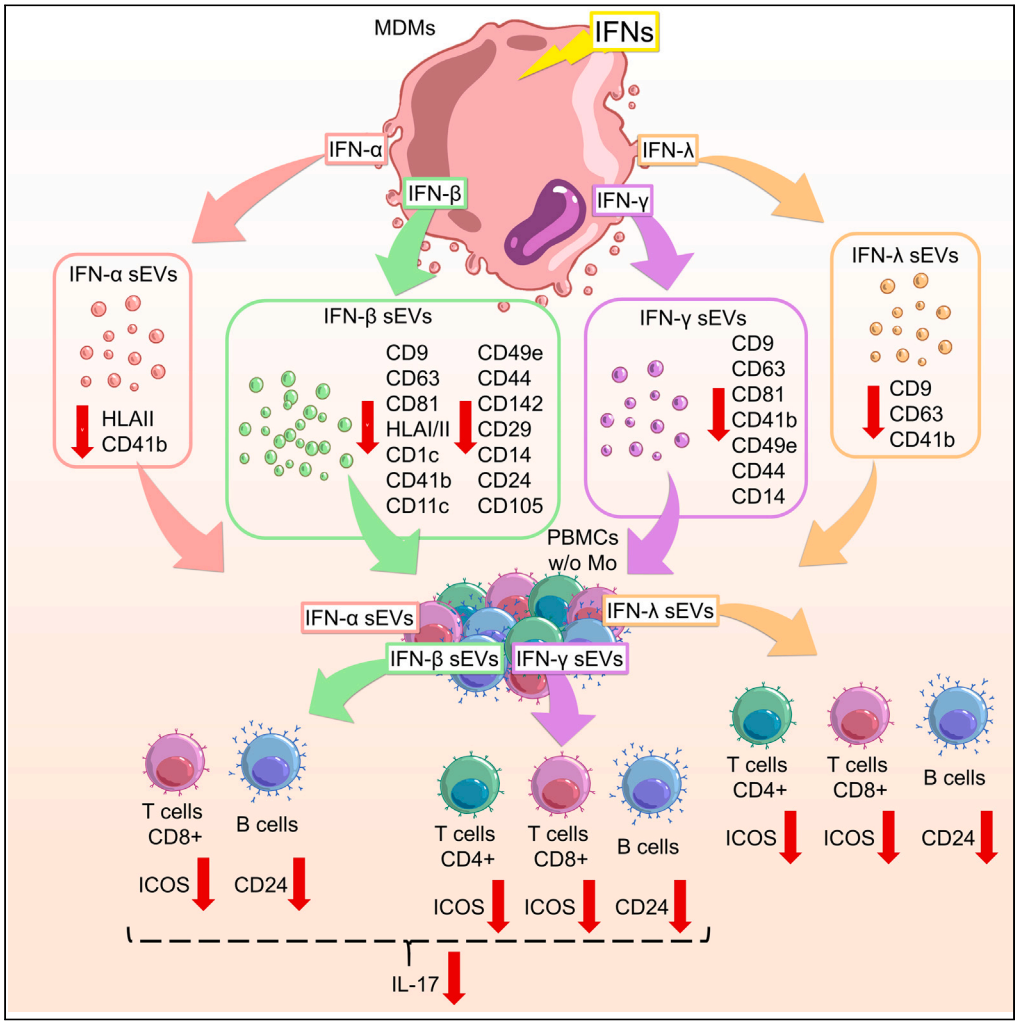

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Highlights

IFNs shape differentially the phenotype of sEVs release by MDM

IFNs-derived sEVs modulate T and B lymphocytes phenotype

IFN-γ/β-induced sEV modulate IL-17 release by T lymphocytes

Giannessi et al., iScience 27, 109960
June 21, 2024
https://doi.org/10.1016/j.isci.2024.109960

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Macrophages treated with interferons induce different responses in lymphocytes via extracellular vesicles

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SUMMARY

Limited information exists regarding the impact of interferons (IFNs) on the information carried by extracellular vesicles (EVs). This study aimed at investigating whether IFN-α2b, IFN-β, IFN-γ, and IFN-λ1/2 modulate the content of EVs released by primary monocyte-derived macrophages (MDM). Small-EVs (sEVs) were purified by size exclusion chromatography from supernatants of MDM treated with IFNs. To characterize the concentration and dimensions of vesicles, nanoparticle tracking analysis was used. SEVs surface markers were examined by flow cytometry. IFN treatments induced a significant down-regulation of the exosomal markers CD9, CD63, and CD81 on sEVs, and a significant modulation of some adhesion molecules, major histocompatibility complexes and pro-coagulant proteins, suggesting IFNs influence biogenesis and shape the immunological asset of sEVs. SEVs released by IFN-stimulated MDM also impact lymphocyte function, showing significant modulation of lymphocyte activation and IL-17 release. Altogether, our results show that sEVs composition and activity are affected by IFN treatment of MDM.

INTRODUCTION

IFNs are a group of proteins that play a crucial role in the immune system's response to viral infections and to other pathogens.[1–3] There are three types of IFN: type I, type II, and type III. The three IFN types bind to three different specific receptors in mammals and use JAK-STAT as the main signal transduction pathway. They induce common but also cell type-specific effects with intersections of the signal transduction pathways depending on the cell type and the environment present in the different tissues and organs.[4–6]

Type I IFNs (IFN-I), in particular alpha and beta IFN (IFN-α and IFN-β, respectively) are produced by nucleated cells. They are involved in the multi-level regulation of antiviral and antitumoral responses and are considered to be the first line of defense against viral infections, activating antiviral pathways in neighboring cells, and preventing viral replication and spread. Moreover, they enhance the activity of natural killer (NK) cells and macrophages to eliminate infected cells. IFN-α also stimulates the adaptive immune response by promoting the maturation of dendritic cells and the activation of T cells.[7]

Type II IFN (IFN-II), also known as gamma interferon (IFN-γ) is mainly produced by activated T cells, natural killer (NK) cells, and some other immune cells. It is primarily involved in regulating immune responses against intracellular pathogens, such as viruses, fungi and bacteria through the enhancement of the activity of macrophages and the promotion of their phagocytic and microbicidal functions. Moreover, it stimulates the production of antibodies and enhances antigen presentation to T cells playing a crucial role in the adaptive immune response, particularly in Th1 cell differentiation.

Type III IFN (IFN-III), also known as lambda interferon (IFN-λ) is the most recently identified type of IFNs (2003)[8,9] and is produced by various cell types, including epithelial cells and immune cells. It exhibits tissue-specific effects, mainly at mucosal surfaces, and plays a role in the defense against viral infections at those sites inducing antiviral responses in neighboring cells, like IFN-I.[10] The interferon-λ (IFN-λ) family of cytokines consists of interleukin-28A (IFN-λ2), IL-28B (IFN-λ3), and IL-29 (IFN-λ1). The receptor of IFN-λ comprises the IL-28 receptor α-chain (IL-28RA) and the IL-10 receptor 2 chain (IL-10R2). Whereas IL-10R2 is ubiquitously expressed, IL-28RA is more restricted, and expression of this receptor chain has been reported by plasmacytoid DCs, B cells, epithelial cells, and hepatocytes. IL-28RA is not expressed by human primary monocytes and monocyte-derived DCs, but it is expressed by monocyte-derived macrophages (MDMs) rendering them susceptible to IFN-λ[11]

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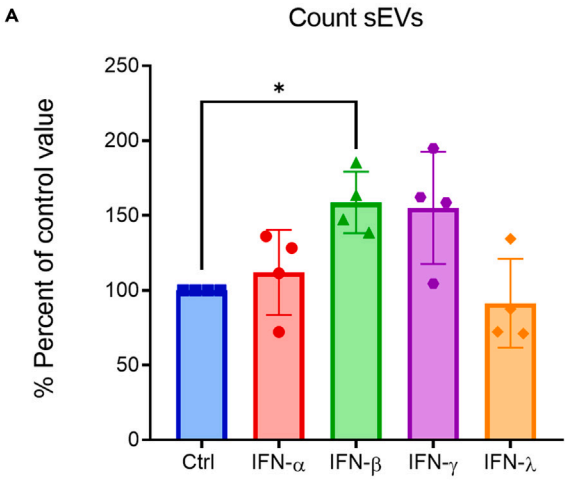
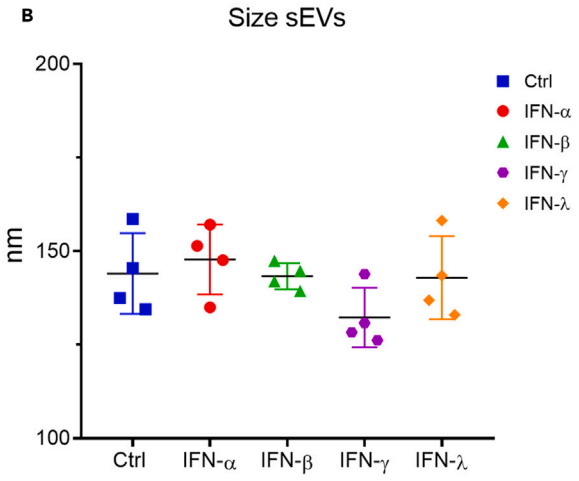
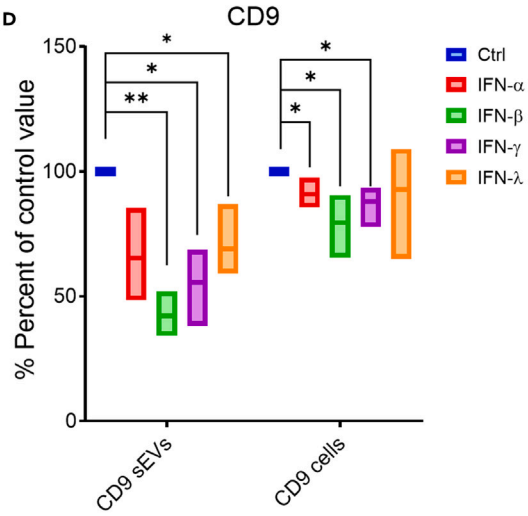
https://doi.org/10.1016/j.isci.2024.109960





C

	IFN-α		IFN-β		IFN-γ		IFN-λ	
	sEVs	cells	sEVs	cells	sEVs	cells	sEVs	cells
HLAI		↑*	↓**					
HLA II	↓*		↓**			↑*		
CD1c			↓**					
CD9		↓*	↓**	↓*	↓*	↓*	↓*	
CD63			↓**		↓*		↓*	
CD81			↓**		↓*			
CD41b	↓*		↓***		↓**		↓**	
CD11c			↓*					
CD49e			↓*		↓*			
CD44			↓**		↓*			
CD142			↓*					
CD29			↓**					
CD14			↓***		↓*			
CD24			↓**			↑**		
CD105			↓**					
CD40				↑*		↑*		



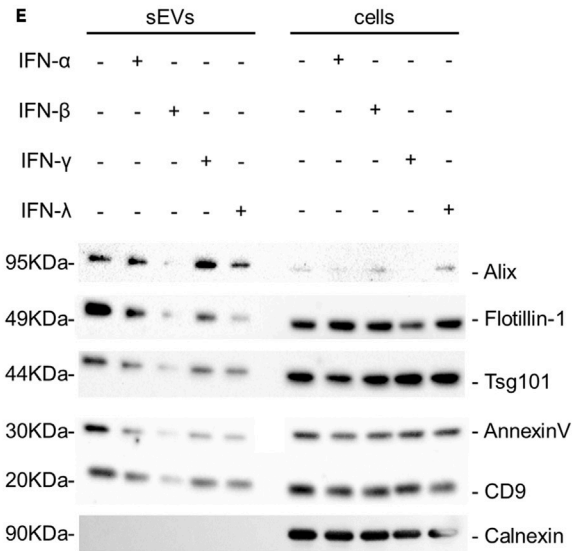


Figure 1. Interferons modulate protein expression levels on sEVs and on macrophages differently

(A) Histogram illustrating the percentage variation in the number of vesicles released by MDM when stimulated with various interferons compared to control (sEVs produced by untreated MDM). Data are expressed as mean ± SD.

(B) Mean diameter of sEVs released from MDM in response to stimulation with interferons. Data are expressed as mean ± SD.

(C) Schematic representation of changes in marker expression within sEVs and MDM-producing cells assessed as a percentage variation relative to the control. Red arrows indicate marker downregulation, while blue arrows indicate marker upregulation. Refer to Figures S1 and S2 for additional details.

(D) Percentage variation compared to control of CD9 in both sEVs and producing cells. Data are expressed as mean ± min-max.

(E) Western blot analysis conducted to detect the presence of various exosomal markers (Alix, Flotillin-1, Tsg101, Annexin V, and CD9) and calnexin as negative marker in the collected sEVs (left; 1.6 μg protein extract) and in the producing cells (right; 25 μg protein extract). The sample was obtained by pooling the sEVs collected to obtain a sufficient amount of protein for the assay. Figure S3 for additional details. The *p*-values were determined using the RM one-way ANOVA or Friedman's test, depending on data distribution. n = samples from 4 healthy donors.

On the whole, interferons (IFNs) type I, II, and III are essential components of the immune system's antiviral response. They regulate immune cell activity, promote antiviral defenses, and help orchestrate an effective immune response against pathogens and cancer. However, vast areas related to IFN biology are still unexplored, for example little is known about their effects on the messages conveyed by extracellular vesicles (EVs). EVs are structures enclosed by membranes, released by all cells in the blood or in other biological fluids. They contain cytoplasm and have the same membrane topology as parental cells, so that they can be considered miniature versions of the donor cell. Typically, EV subpopulations are subdivided, based on their size and biogenesis, into specific categories: small EVs (including exosomes, 40–180 nm in diameter, sEVs), medium/large EVs (microvesicles MV, 100–1000 nm and apoptotic bodies, 1–5 μm).[12,13] These structures are repositories of important information exchanged between cells such as active membrane proteins, lipids and genetic material capable of modulating physiological or pathological processes.[12,14]

Macrophages are the most prolific secretors among innate immune cell types and play a critical role as the first line of defense against infections and diseases.[15,16] Macrophages can secrete and are sensitive to various types of IFNs and the interaction between them is an essential component of the immune response to viral infections. Also, they produce cytokines which modulate the activity and functional differentiation and specialization of adaptive immune cells.[17] Excessive cytokine production, however, can result in tissue damage and autoimmunity, and it is thus tightly regulated.

Here, we show that IFNs can influence the composition, the release and the functions of sEVs released by primary MDMs, thus regulating T cell activity through vesicle-enclosed signals.

RESULTS

Interferons differently modulate protein expression levels on sEVs and on macrophages

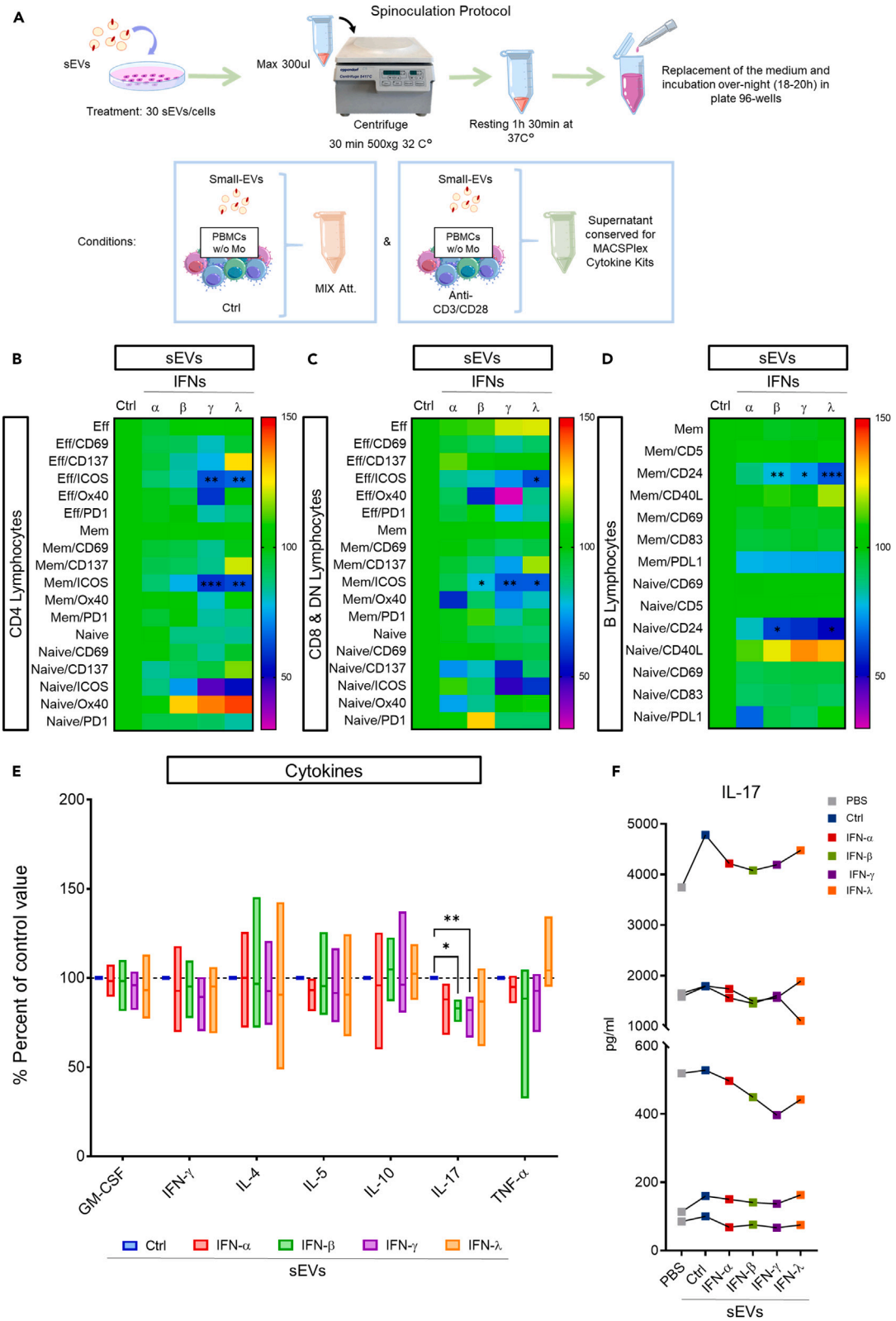
To investigate the effect of different IFNs on sEVs released by in vitro-differentiated macrophages, we isolated vesicles by size exclusion chromatography with the variable size dispersion compatible with the presence of exosomes (range 120–170 nm) from IFN-stimulated macrophage culture supernatants. The number of vesicles released by MDM was significantly increased by 50% compared to the control ($p = 0.027$) only by treatment with IFN-β, while the other IFN treatments did not significantly modify the number of released sEVs (Figure 1A). The number of the producing cells remained constant following the stimulation, indicating that the rise in vesicle number was not due to an increase in the cell count. The size of the vesicles remained unchanged with all treatments (Figure 1B).

The effect of IFNs was mainly observed in the phenotype of the released exosomes. We used the MACS Plex Exosomes Human kit that specifically selects the sEVs that are at least positive for one of the following exosomal markers: the tetraspanins CD9, CD63, and CD81. For this reason, the phenotypic analysis regarded exclusively the exosomes present in our sample. In parallel, we investigated by flow cytometry the expression and the modulation of these markers on the cultured macrophages (Figures 1C and 1D, S1 and S2).

IFN-α and IFN-λ significantly modulated the expression of only a few antigens. In particular IFN-α reduced the expression of class II histocompatibility molecules on vesicles (HLAII, $p = 0.023$) which on the contrary were increased on the cell surface, as was HLA class-I expression ($p = 0.028$). IFN-λ significantly reduced expression of the integrin CD41b ($p = 0.005$) and the tetraspanins CD9 ($p = 0.043$) and CD63 ($p = 0.031$) on sEVs, while it did not affect marker expression on the cells' surface. All the three tetraspanins CD9, CD63 and CD81 were negatively modulated by IFN-β (CD9 $p = 0.002$; CD63 $p = 0.002$; CD81 $p = 0.002$) and IFN-γ (CD9 $p = 0.023$; CD63 $p = 0.032$; CD81 $p = 0.035$) on the sEVs, while only CD9 showed reduced expression on the cells ($p = 0.034$ and $p = 0.03$ respectively); finally, IFN-α did not significantly modify tetraspanin expression on sEVs, but reduced the expression of CD9 ($p = 0.028$) on the cells (Figures 1C and 1D, S1 and S2).

Reduced CD9 expression was validated by western blot analysis (Figure 1E) which confirmed the downregulation of CD9 on both sEVs and cells predominantly after treatment with IFN-β. Due to the small amount of proteins extracted from sEVs, it was preferred to search for other important proteins in the biogenesis process rather than confirming the data on the other tetraspanins CD81 and CD63. In particular, we investigated the modulation of Alix, Flotillin-1, Tsg101, Annexin V, and calnexin (Figures 1E and S3). We found that IFN-β reduced the amount of all these proteins while the other IFNs negatively modulated Flotillin-1, Tsg101 and Annexin V, but not Alix. As expected Calnexin, a protein found in lower abundance in exosomes compared to cells,[18] was absent in the sEV samples.

We then further characterized the phenotype of the sEVs released following stimulation with the different IFNs (Figures 1C, S1 and S2). The effect of IFN-β on the phenotype of the sEVs was broader, significantly modulating the expression of several surface membrane proteins such as CD11c ($p = 0.011$), CD49e ($p = 0.012$), CD44 ($p = 0.002$), CD29 ($p = 0.007$) HLAI ($p = 0.004$), HLAII ($p = 0.004$), CD1c ($p = 0.007$),


A
Spinoculation Protocol
sEVs
Treatment: 30 sEVs/cells
Max 300ul
Centrifuge
30 min 500xg 32 C°
Resting 1h 30min at 37C°
Replacement of the medium and incubation over-night (18-20h) in plate 96-wells
Conditions:
Small-EVs
PBMCs w/o Mo
Ctrl
MIX Att.
&
Small-EVs
PBMCs w/o Mo
Anti-CD3/CD28
Supernatant conserved for MACSPlex Cytokine Kits
B
sEVs
IFNs
Ctrl α β γ λ
CD4 Lymphocytes
Eff
Eff/CD69
Eff/CD137
Eff/ICOS
Eff/Ox40
Eff/PD1
Mem
Mem/CD69
Mem/CD137
Mem/ICOS
Mem/Ox40
Mem/PD1
Naive
Naive/CD69
Naive/CD137
Naive/ICOS
Naive/Ox40
Naive/PD1
150
100
50
C
sEVs
IFNs
Ctrl α β γ λ
CD8 & DN Lymphocytes
Eff
Eff/CD69
Eff/CD137
Eff/ICOS
Eff/Ox40
Eff/PD1
Mem
Mem/CD69
Mem/CD137
Mem/ICOS
Mem/Ox40
Mem/PD1
Naive
Naive/CD69
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Naive/ICOS
Naive/Ox40
Naive/PD1
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IFNs
Ctrl α β γ λ
B Lymphocytes
Mem
Mem/CD5
Mem/CD24
Mem/CD40L
Mem/CD69
Mem/CD83
Mem/PDL1
Naive/CD69
Naive/CD5
Naive/CD24
Naive/CD40L
Naive/CD69
Naive/CD83
Naive/PDL1
150
100
50
E
Cytokines
% Percent of control value
200
150
100
50
0
GM-CSF
IFN-γ
IL-4
IL-5
IL-10
IL-17
TNF-α
Ctrl
IFN-α
IFN-β
IFN-γ
IFN-λ
sEVs
F
IL-17
pg/ml
5000
4000
3000
2000
1000
600
400
200
0
PBS
Ctrl
IFN-α
IFN-β
IFN-γ
IFN-λ
sEVs

Figure 2. Interferon-induced vesicles modulate T- and B-cell phenotype and function

(A) Schematic representation of the protocol used in the experiment.

(B) Heat maps depicting the percentage of phenotypic changes compared to control (sEVs produced by untreated MDM), induced after treatment with sEVs-IFNs, in $CD4^+$ T lymphocytes, (C) $CD8^+$ & double-negative (DN) lymphocytes and (D) B cells. Figures S4 and S5 for additional details.

(E) Percent of variation compared to control of cytokines release by monocyte-depleted peripheral blood mononuclear cells pre-treated with sEVs-IFNs and stimulated through TCR. Data are expressed as mean ± min-max.

(F) IL-17 levels expressed in pg/ml, produced by monocyte-depleted peripheral blood mononuclear cells pre-treated or not (PBS) with sEVs and stimulated through TCR. Each line on the graph corresponds to the levels of IL-17 produced by a single healthy donor. Statistical analysis was performed using RM one-way ANOVA or Friedman's test based on data distribution. n = samples from 6 healthy donors.

CD24 ($p = 0.002$), CD105 ($p = 0.003$), CD142 ($p = 0.019$), and CD14 ($p = 0.0005$), whose expression levels on the vesicles was significantly reduced compared to untreated controls.

On macrophages, IFN-β did not significantly modulate the cell surface expression of these markers, except for CD9 ($p = 0.034$), which was decreased and for CD40 ($p = 0.011$) which was increased.

IFN-γ has a marked effect on both sEVs and cells. On sEVs not only it significantly reduced tetraspanins, but also CD49e ($p = 0.016$), CD44 ($p = 0.048$) and CD14 ($p = 0.017$) while on the cell surface, it decreased the expression of CD9 and increased the expression of CD40 ($p = 0.02$), HLA II molecules ($p = 0.037$) and CD24 ($p = 0.006$). The expression of CD41b was significantly reduced on sEVs by all the IFNs (IFN-α $p = 0.036$; IFN-β $p = 0.0006$; IFN-γ $p = 0.004$; IFN-λ $p = 0.005$).

Thus, these data shows that treatment with IFNs modulates the expression of several proteins both on sEVs and on the cell's surface.

Interferon-induced vesicles modulate the T- and B-cell phenotype

We wondered whether the modulation of the phenotype of macrophage-derived sEVs induced by IFNs might correspond to changes in the messages they carry. To answer this question, we studied the effects of these IFN-induced sEVs on autologous T and B cells. We treated the monocyte-depleted PBMCs with the sEVs (about 30 vesicles per cell) released from autologous macrophages stimulated with the different IFNs. After 18 h of culture with the sEVs, lymphocytes were stained for flow cytometry and analyzed for the expression of markers defining distinct cellular subsets and of several molecules involved in immune activation or co-stimulation (Figure 2A).

SEVs derived from all IFNs treatment, except IFN-α, had the ability to significantly decrease inducible co-stimulator (ICOS) on effector/memory CD4 (effector CD4 Δ = 36%, $p = 0.001$ IFN-γ; effector CD4 Δ = 34%, $p = 0.008$ IFN-λ; memory CD4 Δ = 41%, $p = 0.0002$ IFN-γ; memory CD4 Δ = 38%, $p = 0.006$ IFN-λ) and CD8 T cell subsets (effector CD8 Δ = 36%, $p = 0.012$ IFN-λ; memory CD8 Δ = 22%, $p = 0.035$ IFN-β; memory CD8 Δ = 35%, $p = 0.003$ IFN-γ; memory CD8 Δ = 35%, $p = 0.018$ IFN-λ, Figures 2B and 2C; S4). The sEVs released by unstimulated macrophages did not impact ICOS expression. Thus, it can be inferred that treatment with IFNs modifies vesicles cargo to modulate expression of this co-stimulatory molecule on the cells (Figure S4).

Moreover, sEVs originating from macrophages treated with all IFNs, except IFN-α, notably diminished the expression levels of CD24 on the surfaces of memory and naive B lymphocytes (memory B Δ = 22%, $p = 0.004$ IFN-β; memory B Δ = 26%, $p = 0.019$ IFN-γ; memory B Δ = 36%, $p = 0.0002$ IFN-λ; naive B Δ = 39%, $p = 0.015$ IFN-β; naive B Δ = 49%, $p = 0.012$ IFN-λ). Unlike ICOS, sEVs from untreated macrophages induce a decreasing trend of CD24 on B lymphocytes, but IFNs enhance this capability (Figures 2D and S5). The same experiment was performed using recombinant IFNs directly on lymphocytes, rather than employing IFN-induced vesicles, and it revealed a notably different outcome: IFN-I significantly enhanced the upregulation of CD69 on CD4 T lymphocytes and B cells, while also displaying a tendency to upregulate ICOS on CD4 and CD8 T lymphocytes. (Figures S4 and S5).

Thus, these data show that sEVs released by IFN-stimulated macrophages are biologically active and modulate the expression of surface markers on adaptive immune cells differently from signals induced by IFNs themselves.

Interferon-induced sEVs modulate IL-17 release from T lymphocytes

We then asked whether sEVs also modulate functional abilities such as cytokine release by T cells. To this aim, monocyte-depleted PBMCs were treated with autologous sEVs induced by IFNs and the levels of 7 pro- and anti-inflammatory cytokines were measured after CD3/CD28 stimulation (Figure 2A).

The secretion of cytokines was notably influenced only by sEVs obtained from the supernatants of macrophages treated with IFN-β and IFN-γ, particularly affecting IL-17 release. Specifically, sEVs generated by IFN-β decreased IL-17 production by T cells by 17% ($p = 0.025$) compared to T cells treated with sEVs from unstimulated macrophages, while IFN-γ reduced it by 18% ($p = 0.008$).

Furthermore, sEVs released by untreated macrophages slightly enhanced IL-17 release induced by TCR stimulation alone. However, this effect was significantly attenuated by sEVs released by macrophages treated with both IFN-β and IFN-γ (Figures 2E and 2F).

Direct stimulation with IFNs, specifically IFN-I, leads to an increase in the release of cytokines. These include regulatory cytokines like IL-10, as well as pro-inflammatory cytokines like IFN-γ (Figure S6).

DISCUSSION

The ability of the different classes of IFNs to modulate the immune response through the release of vesicles is a poorly explored field. IFNs are the first molecules to be released by immune cells in response to infections, and macrophages are the cells that intervene first to eliminate the

pathogens. Under these conditions, macrophages are influenced by IFNs that modulate also sEVs production, content and activity. Here we explored the phenotype and activity of sEVs released after 20 h of treatment of human primary macrophages with IFN type I (IFN-α2b and IFN-β), type II (IFN-γ) and type III (IFN-λ1/2).

With the exception of IFN-β treatment, which induced a 50% increase in the amount of sEVs, in general there was no significant increase in the number or dimension of the vesicles released following stimulation with IFNs compared to control macrophages, but we detected significant differences in the protein decoration of these vesicles.

Of the 37 antigens studied on the surface of sEVs, we found 15 proteins to be differentially expressed after IFN-β treatment, 7 after IFN-γ, 3 after IFN-λ and 2 after IFN-α. All of these proteins appeared to be reduced compared to those on sEVs released by untreated macrophages.

The tetraspanins CD9, CD63 and CD81 belong to the group of non-tissue specific EV proteins and are considered to be exosomal marker proteins. According to the MISEV2018 (minimal information for studies of EVs) guidelines[19] they should be present on sEVs even if not necessarily in equal amounts.

We detected a general downregulation of the tetraspannins CD9, CD63 and CD81 on the surface of sEVs mediated by all IFN types except IFNα. This could be attributed to a different biogenesis of sEVs induced by these IFNs. Despite the fact that both IFN-α and IFN-β are IFN-I, bind to the same receptor (IFNAR1/IFNAR2) and signal through similar mechanisms, they have different binding affinities and, consequently, give rise to different antiviral, antiproliferative, and immunomodulatory outcomes. For example, IFN-β has a ∼50-fold higher receptor-binding affinity to IFNAR1 than IFN-α, resulting in a more potent antiproliferative and perhaps distinct immunoregulatory action.[20,21] These differences could account also for the different impacts on EVs.

In 2016, Villarroya-Beltri et al. reported that treatment of the human Jurkat-derived T cell line J77cl20 with 1,000 U/ml of IFN-I for 16 h reduced the levels of expression of the classical exosome markers CD63, TSG101 and CD81 in purified EVs.[22] In that work, the EVs secreted by IFN-treated cells were not only decreased but also were recovered in different fractions than the EVs derived from untreated cells, suggesting that they might be of different nature. Also, a decrease in the number of multivesicular Bodies (MVB) inside the type I IFN-treated cells was reported, suggesting that the EV production derived from MVBs was affected. This effect was likely due to ISG-15, a ubiquitin-like protein markedly induced by type I and III IFNs via transcriptional regulation together with the ISGylation enzyme.[16] It was already known that ISGylation can interfere with the ESCRT machinery, in particular by reducing the retroviral budding and interfering with the interaction of GAG viral protein and the ESCRT component TSG101.[23,24] Indeed, we observed the reduction of Tsg101 and Alix (another ESCRT protein involved in the biogenesis of ILV), but also of Flotillin I and Annexin V, supporting the possibility that IFNs affect the genesis of sEVs, in addition to their content.

Jung at al. also noticed significant differences in the protein surface expression of EVs between healthy subjects and malaria patients.[25] The most modulated proteins were CD106, CD81, osteopontin, HLA-DR and HBEGF on plasma sEVs together with the concentration of thrombocytes. The authors explain the decreased expression of these proteins on plasma sEVs in malaria patients suggesting that immune cells need their receptors for their own activation and do not release them via sEVs. Moreover, the pathway analysis by STRING revealed that the differentially expressed proteins were linked to IFN-γ signaling, crucial during Plasmodium infection.

Our data show that the expression of antigen-presenting molecules (HLA-I, HLA-II, and CD1c) was also negatively regulated on the sEV surface mainly by IFN-β. MHC class I molecules are found on the surface of almost all nucleated cells and present intracellular peptides to CD8 T cells. MHC class II molecules are primarily expressed on antigen-presenting cells, such as macrophages, dendritic cells, and B cells, and are mostly involved in presenting peptides derived from extracellular pathogens to CD4 T cells. The available scientific literature does not provide conclusive evidence that EVs consistently and directly decrease the expression of MHC class I and II molecules on cell surfaces. However, some studies have suggested that EVs derived from certain cell types, such as tumor cells, may affect MHC expression. These effects can vary depending on the specific context, cell types, and stimuli involved. Taylor and Black, for instance, discovered that plasma membrane-derived vesicles shed by metastatic variants of the murine B16 melanoma significantly inhibited the stimulation of macrophage I region-associated antigen system (Ia) expression, the earliest stage in the establishment of an immune response.[26] The presence of antigen presenting molecules on the surface of EVs and their ability to present antigenic peptides to T cells has been well established,[27,28] and the essential role of HLA I in the presentation of peptides to $CD8^+$ T cells provides a promising target for immune therapies, especially in the field of cancer.[4,5] Here, we find that antigen presenting molecules are significantly downregulated on sEVs by IFN-I and at the same time we see a trend for increased levels of expression on macrophages. It is possible that macrophages after stimulation with IFNs tend to keep histocompatibility molecules on their surface in addition to increasing their transcription, preventing their release onto the vesicles.

Another class of molecules that are downregulated on sEVs are adhesion molecules. In our panel, four of these molecules were significantly modulated by IFN-β: CD11c, CD49e, CD44 and CD29.

Accumulating evidence demonstrates that EVs exploit the adhesion molecules to mediate homing to the target tissues, as do cells.[29] For example, integrins have been shown to play important roles in EVs binding to, uptake by, and/or delivery of contents to, target cells.[30]

Two major mechanisms of EVs uptake have been proposed: fusion to the cellular plasma membrane and endocytosis through several different processes.[29,31]

The increased expression of adhesion molecules on vesicles produced by tumor cells has been shown to promote the metastatic dissemination of primary cancer cells to distant organs. Hoshino et all demonstrated that exosomes expressing integrin α6β4 were preferentially distributed to the lung, whereas those expressing integrin αVβ5 were preferentially distributed to the liver.[30] In the non-tumor environment, upon activation, gut trophic lymphocytes have been shown to secrete EVs that express high levels of integrin α4β7, which directs EVs to the gut via binding to MAdCAM-1, which is exclusively and constitutively expressed in the gut.[32] These results underscore the significance of

exosomal regulation of cell homing by modifying the microenvironments of destination tissues. Since we find that IFNs induce sEVs with low integrin expression, it is possible that IFN stimulation determines the release of sEVs intented to act locally at the site of production and not to migrate to distant body sites. It is also possible that other adhesion molecules were upregulated that we did not take into account.

Regarding sEV activity on T lymphocytes, we also found that sEVs induced by all the INFs, except IFN-α, exerted an inhibitory effect specifically on activated T cells.

In particular, we measured a significant downregulation of ICOS on memory and effector populations predominantly in the CD4 T cell subsets. Following T cell activation, the expression of ICOS is upregulated on the cell surface. Additionally, ICOS levels are post-transcriptionally regulated by Roquin, a member of the RING-type ubiquitin ligase family, which facilitates the degradation of ICOS mRNA.[33] This degradation process is reliant on the binding of microRNA-101 to the 3′ untranslated region (UTR) of ICOS mRNA. In sanroque mice, characterized by a mutated Roquin unable to mediate ICOS mRNA degradation, a lupus-like autoimmune disorder ensues due to elevated levels of ICOS.[33] Although we did not observe a correlation between the phenotype of our sEVs and the downregulation of ICOS on lymphocytes, exploring the presence of this microRNA within our sEV samples would be particularly intriguing. In addition, sEVs modulate B cells by decreasing significantly CD24 surface expression. A previous study showed that in CD24-deficient mice dendritic cells are inefficient at priming T cells.[27] Our finding of decreased CD24 levels may thus be in accordance with reduced antigen presentation by B cells. Another plausible hypothesis for the downregulation of CD24 comes from the work of Kedarinath et al. on neuroblastoma cell lines. In this article, the authors demonstrate that cells with low expression of CD24 have a high basal level of an antiviral state, as evidenced by their increased sensitivity to exogenous IFN-I, by constitutive levels of phosphorylated STAT1, and by high expression of antiviral genes such as IRF and NF-κB. This suggests that the neuroblastoma cell line with low CD24 expression is more resistant to viral infection, and ectopic expression of CD24 in this line renders it more permissive to viral infections.[34]

Another sign that IFN-induced sEVs negatively modulate T cell activity is the modulation of IL-17 production. IL-17 is a proinflammatory cytokine synthesized by T helper type 17 cells, which plays a pivotal role in modulating the host immune response against diverse pathogens, including viruses. However, aberrant IL-17 production can trigger chronic inflammation, autoimmunity and contribute to virus-mediated illnesses and virus persistence.[35] Therefore, careful regulation of interleukin-17 production is essential to ensure an effective immune response. Recent research has shown that macrophages can inhibit IL-17 production by T cells through a variety of mechanisms, including the secretion of anti-inflammatory cytokines and the expression of surface molecules that interact with T cells.[36–38] Our finding of a modulation of IL-17 production by sEVs adds another level of regulation of this powerful proinflammatory molecule.

It is conceivable that IFNs, beyond their direct antiviral properties, modulate immune cells by upregulating antigen presentation in antigen-presenting cells and stimulating T and B lymphocytes. This hypothesis finds support in both our data and existing literature, which indicate an upregulation of activating molecules, such as CD69, and an enhanced production of both pro- and anti-inflammatory cytokines upon direct stimulation of T lymphocytes with IFN-I.[2,39–41]This aligns with the organism's imperative to combat an impending infection, with IFNs functioning as an alarm system. Subsequently, IFNs induce immunomodulatory actions, prompting macrophages to generate EVs as negative regulators and conveying negative feedback aimed at dampening the immune response. With this interpretation framework, we can also understand the downregulation of molecules such as CD41b and CD142 on the surface of our sEV induced by IFNs. These molecules are typically activated during inflammation and over-expressed on EVs leading to pro-coagulant activity.[42–44]

It is frequently discussed how mesenchymal stem cells, upon exposure to a proinflammatory environment, secrete immunomodulatory signals into the extracellular space via EVs.[45,46] We posit that various cell types, not limited to mesenchymal stem cells, when exposed to pro-inflammatory stimuli, release signals for modulating immune responses into their surrounding milieu. These signals, likely more complex than those activating receptor molecules, are conveyed through sEVs.

However, further functional and mechanistic studies are needed to clarify the molecules enclosed in sEVs and regulated by IFNs that could participate to the modulation of innate and acquired immunity. Understanding the signals that travel inside the sEVs that control lymphocytes activity may provide new insights and lead to the development of novel therapies for inflammatory and autoimmune diseases.

Limitations of the study

In this study, we characterized the small EVs released by primary human macrophages after stimulation with different types of IFNs. We also assessed the effect these vesicles had on T and B lymphocytes from the same donor. The limitation of this study was our inability to vary the concentration of vesicles, for example, in dose-response experiments, because ultracentrifugation would have altered the isolated vesicles, causing them to merge. Additionally, the limited protein and nucleic acid content in the small vesicles prevented us from investigating their internal cargo.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109960.

ACKNOWLEDGMENTS

This work was supported by FESR (Fondo Europeo di sviluppo regionale- Lazio Innova) GeCoWEB A0375-2020-36641-CUP F85F21003690009. Partially supported by "2023-2027 University Department Excellence Funding Initiative" and partially supoported by the Italian Ministry of Health, "Ricerca Corrente, Linea 2".

AUTHOR CONTRIBUTIONS

D.F.A. conceived and planned the experiments and wrote the manuscript with support from E.A. and G.B.; E.A. and L.B. designed and directed the project; G.B. and Al.S. discussed the results and contributed to the final manuscript; F.G. performed experiments, analyzed all the data including the statistical analysis and assembled the figures; Z.P., An.S. Ve.L. and V.L. performed experiments. E.A., G.B., L.B., and D.F.A. provided funding.

DECLARATION OF INTERESTS

All the authors have no conflicts to declare.

Received: November 30, 2023
Revised: April 9, 2024
Accepted: May 8, 2024
Published: May 10, 2024

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal Anti-Tsg101	Genetex	Cat#GTX70255R
Rabbit monoclonal Anti-CD9	Cell Signaling Technology	Cat#8555
Mouse monoclonal Anti-Alix (3A9)	Cell Signaling Technology	Cat#2171
Rabbit monoclonal Anti-Flotillin-1(D2V7J)	Cell Signaling Technology	Cat#18634
Rabbit monoclonal Anti-Annexin V	Cell Signaling Technology	Cat#8555
Rabbit monoclonal Anti-Calnexin	Promega	Cat#NB100-1974
Anti mouse-HRP	Enzo	Cat#ADI-SAB-100-J
Anti rabbit-HRP	Bio Rad	Cat#1662408EDU
Mouse monoclonal anti CD45 (clone J33) Conjugated FITC	Beckman Coulter	Cat#A07782
Mouse monoclonal anti CD24 (clone ML5) Conjugated FITC	BD Bioscience	Cat#555427
Mouse monoclonal anti CD63 (clone CLBGran/12) Conjugated FITC	Beckman Coulter	Cat#IM1165U
Mouse monoclonal anti CD146 (clone 541-10B2) Conjugated FITC	Miltenyi	Cat#130-126-361
Mouse monoclonal anti CD29 (clone K20) Conjugated FITC	Beckman Coulter	Cat#IM0791U
Mouse monoclonal anti CD49e (clone IIA1) Conjugated PE	BD Bioscience	Cat#555617
Mouse monoclonal anti CD133/1 (clone REA753) Conjugated PE	Miltenyi	Cat#130-110-962
Mouse monoclonal anti CD9 (clone M-L13) Conjugated PE	BD Pharmingen	Cat#555372
Mouse monoclonal anti HLA-ABC (clone W6/32) Conjugated PE	Biolegend	Cat#311406
Mouse monoclonal anti CD105 (clone 43A3) Conjugated PE	Biolegend	Cat#323206
Mouse monoclonal anti CD40 (clone 5C3) Conjugated PE	BD Pharmingen	Cat#555589
Mouse monoclonal anti CD44 (clone BJ18) Conjugated BV421	Biolegend	Cat#338810
Mouse monoclonal anti HLADR-DP-DQ (clone TU39) Conjugated BV421	BD Horizon	Cat#564244
Mouse monoclonal anti CD69 (clone FN50) Conjugated BV421	BD Horizon	Cat#562884
Mouse monoclonal anti CD25 (clone BC96) Conjugated BV421	Biolegend	Cat#302630
Mouse monoclonal anti CD261/TRAIL-R1 (clone S35-934) Conjugated BV786	BD OptiBuild	Cat#744711
Mouse monoclonal anti CD56 (clone NCAM 16) Conjugated BV786	BD Horizon	Cat#564058
Mouse monoclonal anti CD86 (clone 2331) Conjugated BV786	BD OptiBuild	Cat#740990

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse monoclonal anti CD209/DC-SIGN (clone REA617) Conjugated APC	Miltenyi	Cat#130-124-257
Mouse monoclonal anti CD62P (clone AK-4) Conjugated APC	BD Pharmingen	Cat#550888
Mouse monoclonal anti CD81 (clone JS64) Conjugated APC	Beckman Coulter	Cat#A87789
Mouse monoclonal anti CD31 (clone AC128) Conjugated APC	Miltenyi	Cat#130-119-891
Mouse monoclonal anti CD11c (clone REAA618) Conjugated APC	Miltenyi	Cat#130-114-110
Mouse monoclonal anti CD95 (clone DX2) Conjugated FITC	BD Pharmingen	Cat#555673
Mouse monoclonal anti CD154/CD40L (clone TRAP-1) Conjugated PE		Cat#IM2216U
Mouse monoclonal anti CD69 (clone FN50) Conjugated BB700	BD OptiBuild	Cat#747520
Mouse monoclonal anti CD5 (clone UCHT2) Conjugated PE-Cyanine7	Biolegend	Cat#300621
Mouse monoclonal anti CD45RA (clone 2H4) Conjugated PE-Cyanine7	Beckman Coulter	Cat#B10821
Mouse monoclonal anti CD137 (clone 4B4-1) Conjugated BV421	BD Horizon	Cat#564091
Mouse monoclonal anti CD274/PD-L1 (clone MIH1) Conjugated BV605	BD OptiBuild	Cat#740426
Mouse monoclonal anti CD278/ICOS (clone DX29) Conjugated BV605	BD OptiBuild	Cat#745100
Mouse monoclonal anti CD83 (clone HB15e) Conjugated BV650	BD OptiBuild	Cat#740602
Mouse monoclonal anti CD279/PD-1 (clone EH12) Conjugated BV650	BD OptiBuild	Cat#564104
Mouse monoclonal anti CD27 (clone L128) Conjugated BV786	BD Horizon	Cat#563327
Mouse monoclonal anti CD3 (clone UCHT1) Conjugated BV786	BD Horizon	Cat#656491
Mouse monoclonal anti CD134/ox40 (clone ACT35) Conjugated APC	Biolegend	Cat#350008
Mouse monoclonal anti CD3 (clone UCHT1) Conjugated APC-Alexa Fluor 700	Beckman Coulter	Cat#B10823
Mouse monoclonal anti CD4 (clone REA623) Conjugated APC-Vio770	Miltenyi	Cat#130-113-223
Mouse monoclonal anti CD19 (clone REA675) Conjugated APC-Vio770	Miltenyi	Cat#130-113-643
Biological samples		
Human Peripheral Blood Mononuclear Cells (PBMCs)	Buffy coats from Croce Rossa italiana, Italy	N/A
Chemicals, peptides, and recombinant proteins		
Lympholyte-H	Cedarlane Laboratories Ltd., Burlington, ON, Canada	Cat#CL5020

(*Continued on next page*)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RPMI 1640 medium	Sigma-Aldrich, Milan, Italy	Cat. #R0883
L-glutamine	Gibco, Amarillo, TX, USA	Cat#15430614
Penicillin & streptomycin	Sigma-Aldrich, Milan, Italy	Cat#P4333
Fetal Bovine Serum (FBS	Gibco, Amarillo, TX, USA	Cat. #F7524
Beads $CD14^+$	Miltenyi Biotec, Germany	Cat. # 130-050-201
GM-CSF	PeproTech	Cat. #300-03
human recombinant IFN-α2b Intron-A 10 rec-interferon alfa-2b-power for powder for injectable solutions	Schering-Plough SpA, Milan Italy	N/A
human recombinant IFN-β	Ares-Serono, Geneva, Switzerland	N/A
human recombinant IFN-γ	EC Ltd PeproTech, London, UK	Cat. #300-02
human recombinant IFN-λ1	EC Ltd PeproTech, London, UK	Cat#300-02L
human recombinant IFN-λ2	EC Ltd PeproTech, London, UK	Cat#300-02K
anti-CD3-CD28 Dynabeads	Thermo Fisher Scientific	Cat#:11131D
M-PER Mammalian Protein Extraction Reagent	Thermo Scientific	Cat# 78505
HaltTM Protease inhibitor Cocktail EDTA-free-100x	Thermo Scientific	Cat#78425
Acrylamide solution (30 %) - Mix 37.5 : 1 for molecular biology	AppliChem	Cat#A3626
0.45 µm pore size nitrocellulose membranes AmershamTM	GE Healthcare Life Science, Milan, Italy	Cat# 10600002
ECL Fast Pico	Immunological Sciences, Rome, Italy	Cat#ECL-2001
Ponceau SPonceau S Solution for Electrophoresis (0.2 %)	SERVA Serving Scientists	Cat#33427.01
Bio-Rad Protein Assay Dye Reagent Concentrate (Bradford)	Bio-Rad	Cat#5000006
Critical commercial assays		
MACSPLex Human Exosomes kit	Milteny Biotec	Cat#130-108-813
MACSPlex Cytokine kit	Milteny Biotec	Cat#130-101-740
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation	Thermofisher	Cat#L34957
Software and algorithms		
FlowJo v10.6.1	Becton Dickinson	https://www.flowjo.com
Image Lab software	(Bio-Rad)	https://www.bio-rad.com
GraphPad Prism 9 – Version 6.01	GraphPad	https://www.graphpad.com/
Other		
SW41 Ti rotor	Beckman Coulter, Brea, CA, USA	N/A
qEV10/35nm columns	IZON, New Zeland	Cat# IC10-35
CytoFlex cytometer	Beckman Coulter	N/A
Nanosight NS300	Malvern Panalytical, UK	N/A
The ChemiDoc XRS	Bio-Rad, Hercules, CA, USA	N/A
Eppendorf 5301 concentrator	Eppendorf	N/A
SW32 Ti rotor	Beckman Coulter, Brea, CA, USA	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniela F. Angelini, mail: df.angelini@hsantalucia.it.

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Primary monocyte collection and differentiation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy coats obtained healthy donors (HD). PBMCs were isolated with Lympholyte-H (Cedarlane Laboratories Ltd., Burlington, ON, Canada) density gradient centrifugation and maintained in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) supplemented with 2 mM L-glutamine (Gibco, Amarillo, TX, USA), 100 Units/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, Milan, Italy) and 10% fetal bovine serum (FBS) (Gibco, Amarillo, TX, USA), previously inactivated at 56°C for 30 min. Monocytes (Mo) were isolated from PBMCs by positive selection using an immunomagnetic-based beads $CD14^+$ (Miltenyi Biotec, Germany), according to the manufacturer's recommendations. The purified monocytes were differentiated to macrophages using 25ng/ml of GM-CSF (PeproTech) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 Units/mL penicillin, 100 μg/mL streptomycin, 25 mM Hepes and 20% heat-inactivated FBS. Monocytes were seeded equally for each condition between 10 and $19x10^6$ cells per $75cm^2$ flask, according to the yield obtained from each donor. Cells were maintained at 37°C in an incubator with a 5% CO_2 humidified atmosphere. The cells were kept in differentiation for 6 days until use. The negative fraction, PBMCs depleted of monocytes, were frozen and stored at -150°C.

METHOD DETAILS

Vesicle-free serum preparation

To avoid interference due to the presence of EVs in the FBS, a vesicle-free serum was prepared and used to supplement the culture medium. FBS was first centrifuged at 2,000xg for 20 min to remove larger debris and then ultracentrifuged at 110,000xg for 18 h to remove EVs. Ultracentrifugation steps were performed at 4°C using an SW41 Ti or a SW32 Ti rotors (Beckman Coulter, Brea, CA, USA). The collected supernatant was then filtered through a 0.2 μm filter and stored at +4°C until use.

Interferons

The following IFNs were used for *in vitro* stimulation: human recombinant (r) IFN-α2b (Intron A, Schering-Plough SpA;) human rIFN-β (Ares-Serono, Geneva, Switzerland; title 90 x 10^6 IU /ml); human rIFN-γ (cat. #300-02; EC Ltd PeproTech, London, UK); and a mix of human rIFN-λ1 and rIFN-λ2 (50% of each one; hereafter referred to as IFN-λ) (EC Ltd PeproTech, London, UK). IFNs doses were chosen on the basis of dose-response curves performed on primary macrophages. In particular, cells were treated for 15 and 30 min with the different IFNs and cell extract were analysed by western blot for induction of STAT1 tyrosine phosphorylation, one of the transcriptional factor-induced early in interferons signaling pathways. The intensity of P-Tyr-STAT1 versus STAT1 was measured and the smallest dose of IFN capable of markedly increase the phosphorylation of the STAT1 has been chosen. The doses chosen were: IFN-α2b and -β were used at 500 IU/ml, IFN-γ at 5ng/ml and IFN-λ1/λ2 at 50ng/ml.

Isolation and purification of extracellular vesicles

sEVs were collected from the same number of producing cells (mean $14x10^6$ cells each condition), and cultured in the same amount of collection medium harvested after 20 hours. sEV isolation was performed according to the procedure approved by The International Society for Extracellular Vesicles (ISEV).[19] Specifically, the collection medium was centrifuged at 290xg for 7 min to remove cells and then at 2000xg for 20 min to remove cell debris. The supernatants were then subjected to an ultracentrifugation step at 15,000 x g for 20 min performed at 4°C using an SW41 Ti rotor (Beckman Coulter, Brea, CA, USA) to remove medium/large EVs (m/lEVs). The volume of the supernatant was quantified and pre-filtered PBS was added to reach 10ml. sEVs were purified through size exclusion chromatography (SEC) using the qEV10/35nm columns (IZON, New Zeland) according to the instruction manual. The method allows to remove over 99% of contaminating soluble proteins and ensures a high yield of sized vesicles ranging from 35 to 120 nm. The vesicle samples were stored in pre-filtered PBS (total recovery 10ml of PBS resuspended vesicles) at + 4°C and used within 48 hours for subsequent experiments.

Nanoparticles Tracking Analysis (NTA)

Nanoparticles Tracking Analysis (NTA) technology was used to determine the concentration and size of isolated vesicles from 4 HD. All samples were quantified by NTA with a Nanosight NS300 (Malvern Panalytical, UK) equipped with a 532 nm laser. The acquisition was made using a camera level of 15. Five videos of 60 sec were acquired and analysed for each sample. The setting of the analysis software was kept constant for all samples. The samples were used undiluted or diluted (1:2 to 1: 4) in pre-filtered PBS in order to have a concentration suitable for reading, following the recommendations of the user manual.

Flow cytometry

To characterize the in-vitro differentiated macrophages following treatment with IFNs, MDM were collected and labeled with a pre-determined concentration of antibodies listed in key resources table. The phenotype of lymphocytes exposed to sEVs derived from MDM was determined through staining with two optimized antibody panels. The panels include antibodies that recognize antigens important for dividing CD4 and CD8 T cell populations into: T naïve ($CD27^+$ CD45RA); T effectors (CD27neg CD45RAneg) and T memory ($CD27^+$CD45RAneg). B lymphocytes, on the other hand, have been classified as memory or naïve based on the expression of CD27.

MDM samples were acquired with a 3 laser/15 parameter CytoFlex cytometer (Beckman Coulter), while lymphocyte samples were analysed with a 6 laser/23 parameter CytoFlex XL. For each sample, approximately 500,000 cells were selected based on scatter parameters, and the analysis was conducted after the exclusion of dead cells and coincident events. The data were analysed using FlowJo v10.6.1 (Becton Dickinson).

MACSPLex exosomes human kit

The membrane biomarkers of the small-EVs isolated from 4 HD were analysed using the MACSPLex Human Exosomes kit (Milteny Biotec). This method allows to identify 37 different exosomal membrane epitopes and two control isotypes. The kit includes the following markers: CD3, CD4, CD19, CD8, HLA II, CD2, CD1c, CD209, CD45, CD20, CD14 markers uniquely expressed by cells of the immune system; CD105, CD56, CD44, CD49e, CD62P, CD326, CD11c, CD146, CD29, CD41b, CD42a, CD31, molecules with adhesion and migration functions; HLA I, CD9, CD81, CD63, CD133/1, that are receptors expressed by many cells of our organism involved in different biological processes; CD25, CD69, ROR1, CD142, CD40, CD24, CD86, molecules important for cell activation or inhibition; MCSP and SSEA-4 which are stem cell markers. sEV were used at a concentration of $5X10^8$/ml and the test was performed following the instruction manual. The samples were analysed by flow cytometry.

Treatment of PBMCs depleted monocytes with extracellular vesicles or interferons

Cell treatments with sEVs were performed on 6 HD. EVs-free FBS both in the collection medium and in the treatment medium, to avoid the possible interference due to the presence of vesicles in the serum. The treatment of cells in suspension with sEVs followed a procedure named spin-inoculation, usually used in viral infection. To favour EVs-cells contact and entry, $3,6 x10^6$ cells were seeded in a 1,5 ml eppendorf tube for each condition. Then a volume of 30 sEVs/cells in pre-filtered PBS was added to the cells. All the samples were brought to a final volume of 300ul with pre-filtered PBS and 10% of free-EVs FBS, and centrifuged at 500 x g 32°C for 30 minutes. The samples were maintained in the incubator at 37°C for 1,5 hours without suspending the pellet and then the cell were plated in two conditions: with or without anti-CD3-CD28 Dynabeads (Gibco). After 18h of incubation at 37°C 5%CO_2, cells unstimulated through TCR were stained with several mouse anti-human antibodies for the markers listed in key resources table while supernatants from anti-CD3-CD28 stimulated samples were collected for cytokine detection. We measured 7 cytokines (GM-CSF, IFN-γ, IL-4, IL-5, IL-10, IL-17, TNF-α) using the MACSPlex Cytokine kit (Myltenyi Biotec). PBMCs depleted of monocytes from 3 HD were treated directly with recombinant IFNs to assess their effect on. We followed the same protocol as used for treating the cells with vesicles.

Western blot analyses

sEVs after SEC purification were pelleted through ultracentrifugation at 110'000x g for 3h. The ultracentrifugation steps were performed at 4°C using an SW41 Ti rotor (Beckman Coulter, Brea, CA, USA). After the ultracentrifugation the supernatant was discarded, and the pellet was left in ice for 15 min before the resuspension. The vesicle samples were concentrated in Speedvac (Eppendorf 5301 concentrator) until a final volume of 5-10 ul was reached. The samples were lysed using the M-PERM 1X lysis buffer (M-PER Mammalian Protein Extraction Reagent, Thermo Scientific) supplemented with a cocktail of protease inhibitors (HaltTM Protease inhibitor Cocktail EDTA-free-100x, Thermo Scientific). The samples were lysed for 30 min in ice and then stored at -80°C.

The protein concentration of vesicles extract was determined by the Bradford protein quantification assay. The samples were then resolved by 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting onto 0.45 μm pore size nitrocellulose membranes (AmershamTM, GE Healthcare Life Science, Milan, Italy) overnight at 35 V using a Bio-Rad Mini Trans-Blot Cell. After blocking in 3% BSA in TTBS/EDTA, membranes were washed twice with TTBS/EDTA for 10 minutes and incubated overnight at 4°C with specific primary antibodies recognizing: Tsg101 (Genetex); CD9 (Cell signaling); Alix (Cell signaling); Flotillin-1 (Cell signaling); Annexin V (Cell signaling); Calnexin (Promega). After washing the membranes were incubated for 1h at room temperature with secondary antibodies conjugated with horseradish peroxidase (HRP). The ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) instrument and the Image Lab software (Bio-Rad) were used to reveal the chemiluminescence signal produced by enhanced chemiluminescence reaction (ECL Fast Pico; Immunological Sciences, Rome, Italy). The membranes were stained with Ponceau S solution before the incubation with the specific antibodies to verify the protein distribution and to use the obtained profile for normalization.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Differences were statistically evaluated using the Repeated Measures (RM) one-way ANOVA or Friedman test with GraphPad prism, considering the type of distribution, Normal or Not-normal respectively.

p values ≤ 0.05 were considered statistically significant (* $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$). All data are expressed as the mean percentage increase vs the control (untreated) cells.