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Comparison of the effects of bacteriophage-derived dsRNA and poly(I:C) on *ex vivo* cultivated peripheral blood mononuclear cells

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ABSTRACT

Double-stranded RNA (dsRNA), regardless of the origin and nucleotide sequence, exhibits multiple biological activities, including the establishment of an antiviral state and modulation of the immune response. Both involve the stimulation of innate immunity primarily via the release of pro-inflammatory cytokines, which in turn shapes the adaptive immune response. In this study, we compared the immune response triggered by two different dsRNAs: 1) a well-known synthetic dsRNA–poly (I:C); and 2) bacteriophage-derived dsRNA (bf-dsRNA) that is a replicative form of ssRNA bacteriophage f2. Human peripheral blood mononuclear cells (PBMCs) from 61 heathy volunteers were stimulated *ex vivo* with both dsRNAs. Subsequently, activation markers on the main lymphocyte subpopulations were analysed by flow cytometry and the production of 29 different cytokines and chemokines was measured by Luminex xMAP technology. The effect of bf-dsRNA on *ex vivo* cultivated PBMCs is similar to that induced by poly(I:C), albeit with subtle dissimilarities. Both treatments increased expression of the lymphocyte CD38 marker and intracellular IFN-γ in $CD8^+$ T and natural killer (NK) cells, as well as the CD95 marker on the main lymphocyte subpopulations. Poly(I:C) was a stronger inducer of IL-6, IL-1β, and CCL4, whereas bf-dsRNA induced higher levels of IFN-α2, CXCL10, and CCL17. These differences might contribute to a distinct clinical manifestation when used as vaccine adjuvants, and bf-dsRNA may have more profound activity against several types of bacteria.

1. Introduction

Double-stranded RNA (dsRNA) as a molecular structure is often associated with viral infections. This is because the genetic material of some viruses exists in the form of dsRNA and dsRNA intermediates are often produced during viral replication [1]. Due to its physiological importance, the cellular and molecular mechanisms by which dsRNA activates the immune system have been actively investigated.

Polyriboinosinic:polyribocytidylic acid (poly[I:C]) is a synthetic dsRNA that mimics the effects of naturally occurring dsRNA. For decades, poly(I:C) has been extensively investigated for its immune-stimulatory properties and its potential use as a vaccine adjuvant [2]. Although several poly(I:C) modifications [e.g., poly(IC:LC) and poly ($IC_{12}U$)] [3] have been developed, none of these derivatives have passed any clinical trials, primarily due to the range of endotoxin-like side effects [4,5]. Thus, there is a continued search for potential dsRNA analogues.

Bacteriophage-derived dsRNA (bf-dsRNA), also known as Larifan (Larifan Ltd., Riga, Latvia), is a heterogeneous population of dsRNA, which have been isolated from *E.coli* cells infected with a single-stranded RNA bacteriophage f2 mutant [6,7]. Larifan is best known for its ability to induce type (I) IFNs and is therefore used as an antiviral agent for the treatment of herpes, papilloma, and influenza virus infections [7]. Nonetheless, Larifan has also been shown to exhibit antitumoral effects in experimental tumour models and several preliminary clinical investigations with cancer patients without inducing serious side effects [7]. However, the effect of Larifan has not been analysed systemically. In this study, for the first time, we directly compared the immunomodulatory effects triggered by bf-dsRNA and poly(I:C) on freshly isolated human peripheral blood mononuclear cells (PBMCs) in

Abbreviations: dsRNA, double-stranded RNA; bf-dsRNA, bacteriophage-derived double-stranded RNA; poly (I:C), polyriboinosinic:polyribocytidylic acid; PBMCs, peripheral blood mononuclear cells; NK, natural killer cells; Th, T helper cell; Tc, cytotoxic T cell; DC, dendritic cells; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; CTLA-4, T-lymphocyte-associated protein

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ex vivo cultures.

2. Materials and methods

2.1. Study population and design

The study was conducted at the Latvian Biomedical Research and Study Centre. The study protocol was approved by the Medical Ethics Committee of Institute of Experimental and Clinical Medicine and informed consent was acquired from all study participants. Healthy men and women aged 18—60 years were eligible for participation. Exclusion criteria included any chronic medical condition, as well as clinically significant abnormal laboratory parameters. The study consisted of two phases: 1) a smaller discovery phase, in which we compared the effects of poly(I:C) and bf-dsRNA on PBMCs; and 2) a validation phase, in which we used bf-dsRNA in a larger sample set. In total, 61 randomly selected healthy volunteers [48 females and 13 males with an average age of 36.0 years (± 12.5 years)] were included in the study and their peripheral blood samples were collected by venepuncture. During the discovery phase, nine individuals [five females and four males with an average age 29.1 years (± 6.5 years)] were included for both Luminex xMAP and flow cytometry analyses, as well as seven females that were included [average age 38.6 years (± 12.1 years)] for ELISAs. In the validation phase, an additional 30 [all females with a mean age of 41.8 years (± 13.6 years)] and 15 [six females and nine males with a mean age of 27.1 years (± 2.8 years)] randomly selected healthy volunteers were included for Luminex xMAP and flow cytometry analysis, respectively.

2.2. PBMC isolation and stimulation

Immediately after blood collection, density gradient centrifugation (2500 rpm for 20 min, Eppendorf 5810R) was performed. PBMCs were then re-suspended in RPMI-1640 growth medium enriched with 10% foetal calf serum and 100 U/mL penicillin and streptomycin (all Sigma-Aldrich, USA) and cultivated at a density of 1×10^6 to 2×10^6 cells/mL at 37 °C in 5% CO_2 atmosphere for 24 h. After 24 h, baseline samples (0 h) were collected. Subsequently, bf-dsRNA (Larifan Ltd, Latvia) or poly(I:C) (Sigma-Aldrich, USA) were added at a concentration of 200 μg/mL as determined during previous experiments [8]. The control cells were cultivated under the same conditions, but did not receive the addition of any stimulant.

2.3. Luminex xMAP® technology

Supernatants were collected at 48 h, 72 h, and 120 h (discovery phase) and 48 h and 120 h (validation phase) following stimulation with dsRNAs and kept frozen at −70 °C until further analysis. The concentrations of 29 different cytokines and chemokines were measured by the Luminex® 200™System using three *Milliplex MAP Multiplex Assay Kits* (Millipore, USA): 1) Cat. # MPXHCYTO-60 K (IFN-α2, IL-17, GM-CSF, CXCL10, TNF-β, IL-3, CCL4, IL-9, and IL-15); 2) Cat. # MPXHCYP2-62 K (CCL17, CXCL12, CCL13, IL-33, IL-28A, IL-23, IL-21, IL-16, CCL1, and CCL27); and 3) Cat. # HSCYTO-60SK (TNF-α, IL-6, IL-5, IL-4, IL-2, IFN-γ, IL-1β, IL-12p70, IL-13, and IL-10). All standards, quality controls, and samples were prepared and measured in duplicate according to the manufacturers' specifications. Results were considered valid if the individual analyte bead count in the sample was > 35 and the variation coefficient between the repeated measures was < 30%.

2.4. ELISA

A commercially available ELISA kit was used to measure IL-10 (R&D Systems, USA). Supernatants were collected at 4, 6, 8, 10, 12, 24, 36, and 50 h after the addition of the stimulant. All standards and samples were prepared according to manufacturer's specifications and measured in duplicate at 450 nm with 540 nm or 570 nm wavelength corrections (BioTek FLx800, USA). The cytokine concentration was calculated using the obtained optical density (O.D.) values with a four-parameter logistic (4-PL) fit.

2.5. Flow cytometry

PBMCs were collected 3 and 10 days after stimulation for both the discovery and validation phases. The collected cell samples were labelled with 18 different fluorophore-conjugated monoclonal antibodies for the following surface markers: anti-CD45-V500, anti-CD8-V450, anti-CD25-V450, anti-HLA-DR-FITC, anti-CD38-FITC, anti-CD4-FITC, anti-CD56-PerCP-Cy™5.5, anti-CD3-APC-H7, anti-CD28-APC, anti-CD95-APC, anti-CD152-PE, anti-CD119-PE (all from BD Biosciences, USA), anti-CD16- PerCP-Cy™5.5, and anti-CD19-PE (both from Santa Cruz Biotechnology, USA). The following intracellular markers were also used: anti-Perforin-PE, anti-Granzyme B-FITC, anti-IFN-γ- Alexa Fluor 647 (all from BD Biosciences, USA), and anti-FoxP3-Alexa Fluor 647 (Santa Cruz Biotechnology, USA). Additionally, the *BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution kit with BD GolgiStop™* (BD Biosciences, USA) was used for Perforin, Granzyme B, and IFN-γ intracellular staining, as well as *Human FoxP3 Buffer Set* (BD Biosciences, USA) for FoxP3 staining. Samples were analysed using a three laser BD FACS Aria II flow cytometer. At least 20×10^3 events were collected. Lymphocytes were gated based on their forward and side-scatter profile. Dead cells and debris were excluded by scatter gates. The data was obtained using BD FACS Diva™ 6.0 (BD Biosciences, USA) software and analysed in FCS Express 6 Flow Cytometry (De Novo Software, Inc., USA). Fluorescence compensations were calculated using a built-in BD FACS Diva™ 6.0 automatic compensation calculator.

2.6. Statistical analysis

All data are displayed as the means ± 95% confidence interval (CI), unless stated otherwise. The statistical significance of the differences among the groups at different time points was determined using a two-way ANOVA for repeated measures followed by a Tukey's multiple comparison test, and considered significant at an alpha level of 5% ($p < 0.05$). GraphPad Prism v6.0 (GraphPad Software, Inc., USA) software was used to perform the statistical analysis and generate graphs.

3. Results

3.1. Bacteriophage-derived dsRNA and poly(I:C) induced cytokines and chemokines in ex vivo cultivated human PBMCs

A total of 13 out of the 29 tested molecules showed an increase in a concentration greater than 5 pg/mL compared to the control in the presence of either dsRNA in at least one of the analysed time points. For 12 molecules, this increase was statistically significant (Fig. 1). Although there was an extremely high increase in the concentration of IL-10 (up to 120,000 pg/mL), no significance between the dsRNA and control was observed due to the large concentration variation amongst the individuals for both poly(I:C) and bf-dsRNA. Similarly, no statistically significant differences in the concentration of IL-10 between both the dsRNA's used and control samples were observed by ELISA (data not shown).

During the validation phase, the same 12 out of 29 molecules reached statistical significance in at least one of the analysed time points (Supplementary Fig. S1).

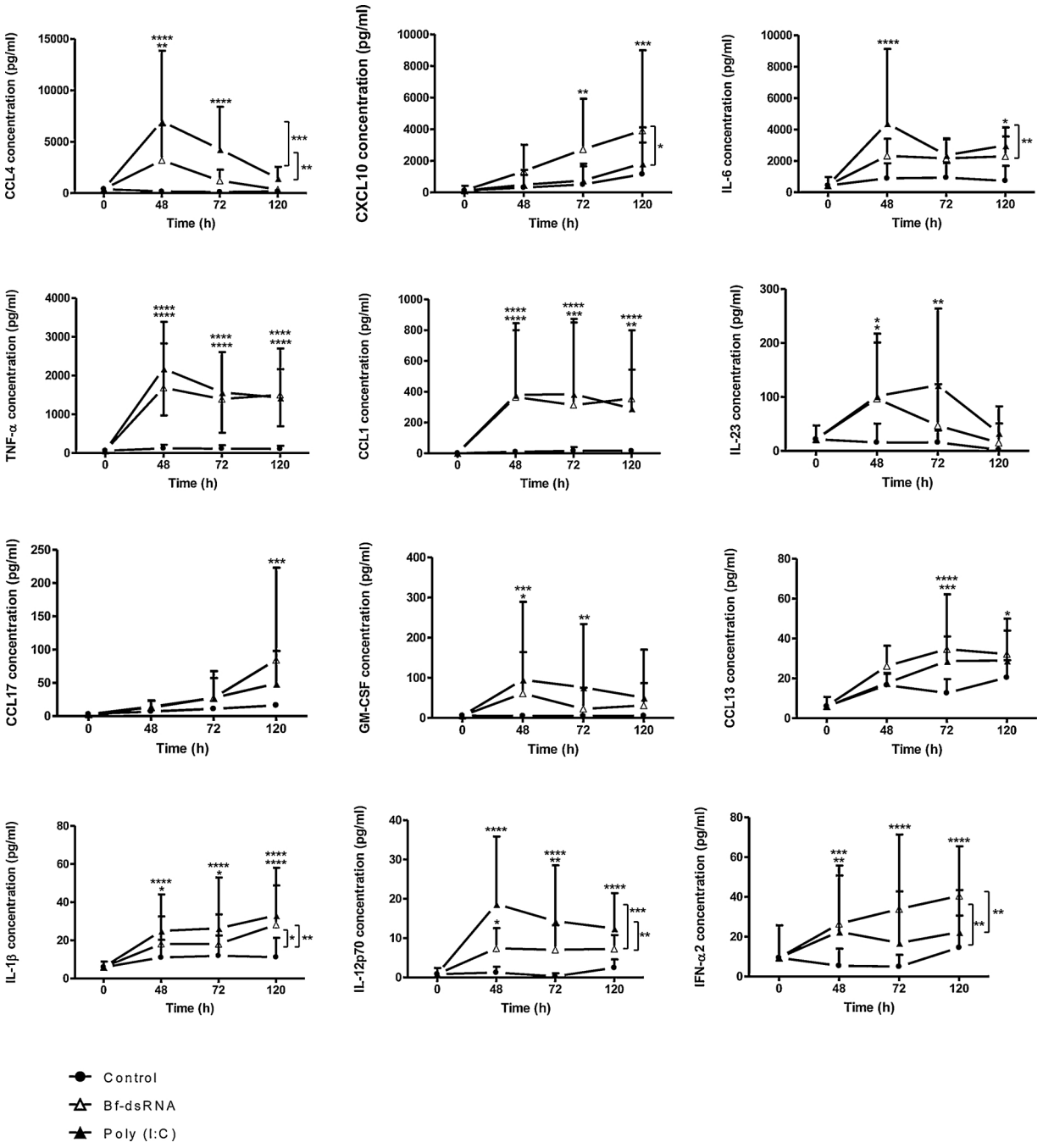

Fig. 1. The cytokine and chemokine stimulatory effect of Poly(I:C) and bf-dsRNA on *ex vivo* cultivated human PBMC (n = 9). The results are displayed as the mean increase + SD with statistical significance, where *represent statistical significance of < 0.05; $^{**}\leq 0.01$; $^{***}\leq 0.001$; $^{****}\leq 0.0001$.

3.2. The effect of bacteriophage-derived dsRNA and poly(I:C) on lymphocyte subpopulations in ex vivo cultivated human PBMCs

Neither of the dsRNAs caused statistically significant changes in the percentage of main lymphocyte subpopulations of T ($CD3^+$), B ($CD19^+$), and NK ($CD16^+CD56^+$) cells. In all samples (bf-dsRNA, poly (I:C), and control groups), a decrease in the percentage of NK cells was observed over time, which was likely due to the culture conditions. In addition, neither treatment with bf-dsRNA nor poly(I:C) caused statistically significant changes in the percentage of cytotoxic T cells ($CD8^+$) or T helper cells ($CD4^+$). However, in the presence of both dsRNAs, CD38 expression and intracellular IFN-γ production was evaluated in cytotoxic T (Tc) and NK cells. The up-regulation of CD38 expression on NK cells showed also a significant difference between both dsRNAs on day 3 ($p < 0.05$) and only for poly(I:C) was significant on day 10 ($p < 0.0001$). In Tc cells, the up-regulation of CD38 expression on day 10 was present only in the case of bf-dsRNA ($p < 0.05$) (Fig. 2). The surface expression of CD152 on T helper (Th) cells was significantly increased only in the case of poly(I:C) ($p < 0.01$). The surface marker CD95 was elevated on all main lymphocyte subpopulations in the presence of both dsRNAs (Fig. 2). The expression of other markers, including HLA-DR, CD25, FOXP3, CD119, and CD28, as well as intracellular granzyme B and perforin did not show any statistically significant changes after PBMCs were stimulated with either of the dsRNAs.

During the validation phase, the statistically significant upregulation of the surface marker, CD95, was found for bf-dsRNA, regardless of the lymphocyte type: T, B lymphocytes, and NK cells (Supplementary

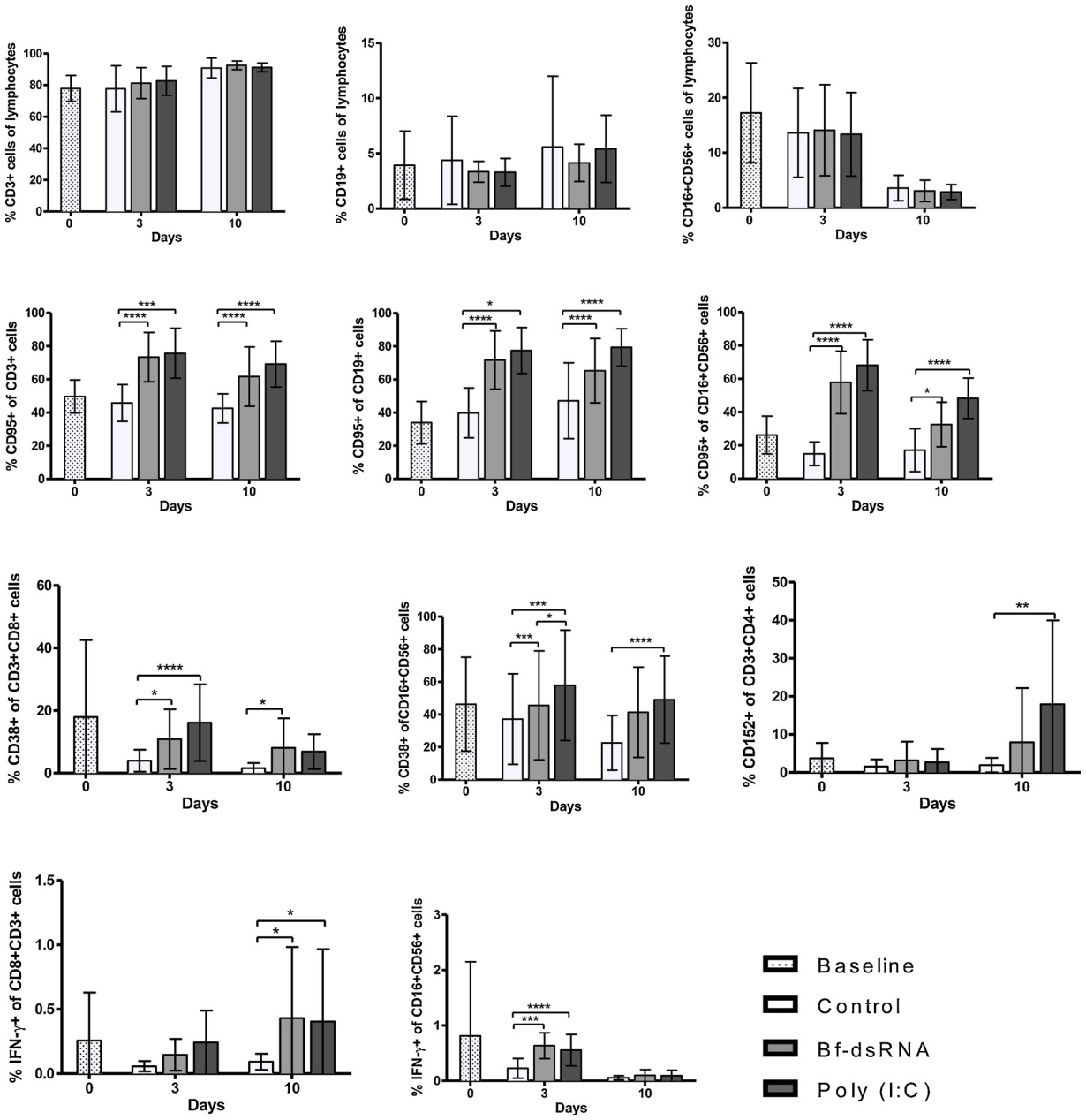

Fig. 2. The effect of bf-dsRNA and poly(I:C) on the expression of lymphocyte molecular markers *ex vivo* (n = 9). The results are displayed as the mean percentage of the cell population with ± 95% CI and the level of statistical significance, where * represents statistical significance of < 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001.

Fig. S2). We also observed an increase in CD38 expression and intracellular IFN-γ production in Tc and NK cells. However, such increases did not reach statistical significance. In addition, we observed that bf-dsRNA induced increased expression of CD152 on Th cells by day 10 ($p < 0.05$) (Supplementary Fig. S2).

4. Discussion

In the present study, we demonstrated that the effect of bf-dsRNA on *ex vivo* cultivated PBMCs was comparable to that of the widely used poly(I:C). Both molecules were able to induce proinflammatory cytokines, which have been shown to link innate and adaptive immunity. In addition, bf-dsRNA appeared to be superior to poly(I:C) in terms of IFN-α induction as it induced higher concentrations of IFN-α over longer period of time. IFN-α is known to exert both antiviral and anti-proliferative effects [9] and is required for $CD8^+$ T cell priming as a third signal for the development of functional T cells [10]. Another difference between the dsRNAs was the level of elevated IL-6, primarily in the presence of poly(I:C). IL-6 is a pleiotropic molecule that also plays a significant role in the production of acute-phase response proteins and the induction of pyrexia during infection [11,12]. Furthermore, a recent study has shown that poly(I:C)-induced fevers in rats can be reduced by intracerebroventricular administration of antibodies against IL-6 [12]. Similarly, the production of IL-1β results in a wide-range of biological changes that are associated with illness (e.g., anorexia, lethargy, and fever) [13]. While IL-1β was induced by both dsRNAs, poly(I:C) induced significantly higher levels of IL-1β. The fact that on average, poly (I:C) induced significantly higher levels of IL-1β and IL-6 than bf-dsRNA may also help explain the differences between the severity of the induced side effects of the two dsRNAs in clinical applications; while poly (I:C) provokes systemic cytokine toxicity [4,5,14] bf-dsRNA does not show serious side effects [7].

Both dsRNA molecules can induce several chemokines and the main differences, again, were found in the induction level of these molecules.

In particular, CCL4 was induced stronger by poly(I:C). CCL4 is known for its ability to suppress viral infections and has been shown to have direct antiviral activity against herpes simplex virus -1 (HSV-1) [15,16]; however, CCL4 is also able to induce a fever [17]. Other chemokines, such as CCL17 and CXCL10, were only significantly induced by bf-dsRNA. Apart from the well-known ability of these chemokines to induce the directed migration of various lymphocyte subsets, both molecules have a direct role in the antimicrobial activity against several types of bacteria (e.g., *Staphylococcus aureus, Streptococcus pyogenes,* and *Bacillus anthracis*) [18,19], demonstrating that bf-dsRNA may also have antibacterial properties. In addition, it has been shown that CXCL10 impairs tumour growth through the recruitment of NK cells and tumour suppressing T cells to enhance the anti-tumoral immune response [20,21].

We also observed an increase in intracellular IFN-γ production in NK and $CD8^+$ T cells by flow cytometry; however, despite this induced intracellular IFN-γ production, we were unable to observe an increase in extracellular IFN-γ. It is possible that the amount of extracellular IFN-γ was too low at the time of measurement (extracellular IFN-γ was measured 120 h after stimulation). Moreover, we observed a decrease in the percentage of NK cells over time and $CD8^+$ IFN-γ producing cells appeared on day 10. Thus, further investigation is required to address this issue. CD38 is another marker that was associated with increased expression on Tc and NK cells. CD38 is a molecule with dual functions as: 1) a cell surface enzyme (ectoenzyme that catalyses the synthesis and hydrolysis of cyclic ADP-ribose); and 2) a receptor, which is involved in cell activation, proliferation, apoptosis regulation, and adhesion [22]. Presumably, CD38 functions as an inducer of NK cell cytotoxicity by stimulating the release of cytotoxic protein granules [23]. In the case of $CD38^+$ NK cells, the expression of CD38 marker differs significantly between poly(I:C) and bf-dsRNA samples. Thus, poly(I:C) appears to be superior over the bf-dsRNA; however, it is possible that the activity of NK cells is again connected with the severity of poly(I:C)-induced side effects, which may be toxic [24]. This hypothesis should also be addressed further in future studies. In the case of $CD8^+$ T cells, the expression of CD38 was increased in the presence of both RNAs; however, it was expressed longer for bf-dsRNA. It has been shown that poly(I:C) decreases $CD8^+$ T cell apoptosis and enhances antigen-specific $CD8^+$ T cell responses [25]. Therefore, it is possible that bf-dsRNA might be able to enhance the antigen-specific $CD8^+$ T cell response and maintain it for a longer period of time. However, additional antigen-specific responses should be analysed. Throughout the experiment, there was increased CD95 expression on all of the lymphocyte subpopulations in the presence of both dsRNAs. The early view on the role of CD95 on immune system functionality postulated that CD95 is involved in the maintenance of homeostasis by preventing excessive activation [26]. Later experiments have demonstrated that, in addition to the induction of apoptosis, signalling via the CD95 receptor is necessary in a wide range of other biological processes, including cellular proliferation, differentiation, migration, and cytokine production [27]. Hence, the ability of dsRNAs to promote CD95 marker expression can be considered both a homeostasis regulatory mechanism upon cellular activation, as well as a necessity for general lymphocyte survival and proliferative signal transduction maintenance. CD152, also known as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), is another marker that displayed increased expression on $CD4^+$ T cells. CTLA-4 is a marker with dual functionality, which typically down-regulates the immune response and maintains homeostasis in several ways (e.g., prevents T cell activation and expansion) [28]. However, despite this function, CTLA-4 also plays a significant role in cell migration and facilitates T cell homing to sites of infection [29].

It is important to note that we observed significant differences in the individual response to these drugs, which must be further addressed with the inclusion and analysis of a greater number of subjects. Moreover, although we have focused primarily on T cell responses, the effect of bf-dsRNA on other cell subpopulations (e.g., macrophages, neutrophils, and eosinophils) and particularly on DCs, should also be addressed.

5. Conclusion

The effect of bf-dsRNA on *ex vivo* cultivated PBMCs was similar to that induced by poly(I:C). Both exhibited the potential to promote the release of proinflammatory cytokines and chemokines *ex vivo*, which could translate to the *in vivo* activation of the innate immune response and subsequent T cell activation. However, subtle dissimilarities existed. For poly(I:C), a stronger induction of IL-6, IL-1β, and CCL4 was related to systemic cytokine toxicity, and a larger increase in the presence of $CD38^+$ on NK cells was observed. In turn, a stronger induction of IFN-α2, CXCL10, and CCL17 was observed for bf-dsRNA, which likely explained why pyrexia is a side effect of poly(I:C) but not bf-dsRNA in a clinical setting. In addition, the latter may have more pronounced antimicrobial activity against several types of bacteria.

Authorship

The study design was formulated by D. Pjanova and S. Donina. Data acquisition was done by R. Petrovska, K. Vaivode, and L. Mandrika. Results analysis and interpretation was performed by K. Vaivode, L. Mandrika, D. Pjanova, and S. Donina. The manuscript was prepared by L. Mandrika K. Vaivode, and D. Pjanova. All authors read and accepted the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.06.010.

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