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Environmental pollutants modulate RNA and DNA virus-activated miRNA-155 expression and innate immune system responses: Insights into new immunomodulative mechanisms

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ABSTRACT

Many persistent organic pollutants, such as polychlorinated biphenyls (PCBs), have high immunomodulating potentials. Exposure to them, in combination with virus infections, has been shown to aggravate outcomes of the infection, leading to increased viral titers and host mortality. Expression of immune-related microRNA (miR) signaling pathways (by host and/or virus) have been shown to be important in determining these outcomes; there is some evidence to suggest pollutants can cause dysregulation of miRNAs. It was thus hypothesized here that modulation of miRNAs (and associated cytokine genes) by pollutants exerts negative effects during viral infections. To test this, an *in vitro* study on chicken embryo fibroblasts (CEF) exposed to a PCB mixture (Aroclor 1260) and then stimulated with a synthetic RNA virus (poly(I:C)) or infected with a lymphoma-causing DNA virus (Gallid Herpes Virus 2 [GaHV-2]) was conducted. Using quantitative real-time PCR, expression patterns for *mir-155*, pro-inflammatory *TNFα* and *IL-8*, transcription factor *NF-κB1*, and anti-inflammatory *IL-4* were investigated 8, 12, and 18 h after virus activation. The study showed that Aroclor1260 modulated *mir-155* expression, such that a down-regulation of *mir-155* in poly(I:C)-treated CEF was seen up to 12 h. Aroclor1260 exposure also increased the mRNA expression of pro-inflammatory genes after 8 h in poly(I:C)-treated cells, but levels in GaHV-2-infected cells were unaffected. In contrast to with Aroclor1260/poly(I:C), Aroclor1260/GaHV-2-infected cells displayed an increase in *mir-155* levels after 12 h compared to levels seen with either individual treatment. While after 12 h expression of most evaluated genes was down-regulated (independent of treatment regimen), by 18 h, up-regulation was evident again. In conclusion, this study added evidence that *mir-155* signaling represents a sensitive pathway to chemically-induced immunomodulation and indicated that PCBs can modulate highly-regulated innate immune system signaling pathways important in determining host immune response outcomes during viral infections.

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Introduction

Persistent organic pollutants (POPs) like polychlorinated biphenyls (PCBs) and their Arochlor (Ars) commercial mixtures are known to interfere with immune signaling pathways (Safe 1994; Olsen 2005). POP production increased until the 1980s when most were banned or regulated on a national basis (Wöhrnschimmel et al. 2016). Nonetheless, these persistent compounds are still found today in the environment and in biota. A peak in the incidence in infectious diseases between 1940 and 2004 was found in the 1980s (74.4% virus and bacteria); this was mainly associated with an increased susceptibility of the host to infections (Jones et al. 2008). These corresponding peaks (pollutants and pathogens) could indicate an involvement of POPs in outbreaks of infectious diseases; this further underlines the need for a closer investigation of the immune system of hosts under continuing exposures to POPs.

The innate immune system represents the first line of defense in a host against pathogens; modulation of these pathways is known to be detrimental (Muralidharan and Mandrekar 2013). Together with cytokines, microRNAs (miRNA) are known to play import roles in innate immune system signaling pathways (Mehta and Baltimore 2016). miRNAs are short, single-stranded non-coding sequences ≈ 22–24 nucleotides (nt) in length that are evolutionary highly conserved and expressed in multicellular organisms as well as in viruses (Bartel 2004; Kozomara and Griffiths-Jones 2011). It has been shown that post-transcriptional modulation by miRNA is involved in regulating ≈ 30% of the human protein genome (Filipowicz et al. 2008), including genes related to immune system function (Brennecke et al. 2003; He et al. 2005; Xiao and Rajewsky 2009).

An important study system to investigate the role of miRNA in infection and disease has been *mir-155* and Gallid herpes virus 2 (GaHV-2; also known as Marek's disease virus [MDV]). MD is caused by chronic GaHV-2 infection in chickens (*Gallus gallus domesticus*), which results in lymphoid tumors. *mir-155* plays an important role in the immune system by regulating cytokine production, T-cell differentiation, T-cell-dependent antibody responses, and B-cell proliferation (Thai et al. 2007). Moreover,

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Supplemental data for this article can be accessed here.

Table 1. Primers used in assays.

NF-κB1	Sense	5′GCAACTATGTTGGACCTGCAAA′	Ghareeb et al. (2013)
	Anti-sense	5′ACCCACCAAGCTGTGAGCAT′	
28S rRNA	Sense	5′GGTATGGGCCCGACGCT′	Neerukonda et al. (2016)
	Anti-sense	5′CCGATGCCGACGCTCAT′	
IL-4	Sense	5′GAGAGGTTTCCTGCGTCAAG′	Xing et al. (2008)
	Anti-sense	5′TGGTGGAAGAAGGTACGTAGG′	
IL-8	Sense	5′CTGGCCCTCCTCCTGGTT′	Ghareeb et al. (2013)
	Anti-sense	5′GCAGCTCATTCCCCATCTTTAC′	
TNFα	Sense	5′CCCCTACCCTGTCCCACAA′	Ghareeb et al. (2013)
	Anti-sense	5′TGAGTACTGCGGAGGGTTCAT′	

mir-155 is substantially induced by the activation of the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (*NF-κB*) signaling pathway in response to toll-like receptor (TLR) signaling (Mehta and Baltimore 2016). Interestingly, GaHV-2 is known to replace and down-regulate *mir-155* in host cells by its own ortholog (miR-M4) to utilize *mir-155* pathways (Morgan et al. 2008; Zhao et al. 2009).

The current study thus focused on the chicken/GaHV-2 system using chicken embryo fibroblasts (CEFs) to investigate combined effects of exposure to POPs and virus infection on the expression of immunologically relevant genes and *mir-155*. CEFs have been shown to be competent in inducing various genes that encode for proteins involved in inflammation, as well as miRNA, upon GaHV-2 infection (Burnside et al. 2006; Burnside and Morgan 2007). Therefore, CEFs are widely used as suitable models for GaHV-2 infections (Haunshi and Cheng 2014; Hu et al. 2016). By analyzing the expression profile of *NF-κB1*, tumor necrosis factor (*TNF*)-α, *IL-8*, and *IL-4* (mRNA), and *mir-155* in response to GaHV-2 or to the synthetic viral double-stranded (ds) RNA analog polyinosinic-polycytidylic acid (poly I:C), the present study sought to investigate natural defense mechanisms by the cells as well as differences between a RNA virus analog and an actual dsDNA virus infection.

Materials and methods

Cell culture, exposure to Ar1260, virus intermediate inoculation, and GaHV-2 infection

All experiments were conducted with CEFs (ATCC CRL12203, *Gallus gallus* embryo, spontaneously-transformed) of the same passage to ensure comparable results. The CEF were cultivated in Dulbecco's modified Eagle's medium (DMEM, Sigma, Oslo, Norway), 5% fetal calf serum, 10 µg gentamycin/ml, 100 units penicillin/ml, and 100 U streptomycin/ml (complete growth medium) (Castaño-Ortiz et al. 2019). The cells were seeded at a density of 5.2×104 cells/ml in 96-well plates and incubated at 39 °C with 5% CO_2 for 48 h. Thereafter, the treatment groups were cells exposed to Ar1260 (22.23 ppm dissolved in corn oil; this concentration was chosen based on preliminary experiments done to attain a sublethal concentration in the CEF) and control cells that received vehicle only. Corn oil was used instead of dimethyl sulfoxide (DMSO) due to both the known immunomodulating as well anti-viral actions of DMSO (Aguilar et al. 2002; Timm et al. 2013).

After 24 h, the supernatant of each culture was removed and a 1:10 dilution of GaHV-2-infected CEF stock (ATCC VR-2175) was added to half of the cultured CEFs that had undergone the Ar1260 (or vehicle) pre-exposure. The other half of the respective treatment group cultures received only complete medium in place of the GaHV-2-infected CEF stock to account for volume changes in each well. The GaHV-2 dilution used was chosen to ensure sufficient viral action on the CEF (Waugh et al. 2018). The same approach as above was completed with poly(I:C), i.e. after 24 h, the supernatant of each CEF culture was removed and fresh complete medium containing 2 µg poly(I:C)/ml was added - with and without Ar1260 pre-exposure (Waugh et al. 2018). In cultures that did not receive poly(I:C), volume change was compensated for by addition of corn oil to the wells.

In all cases, cells were harvested 8, 12, and 18 h (80, 84, 90 h post-seeding) after GaHV-2 infection or poly(I:C) inoculation by trypsinization (two washes of cells with phosphate-buffered saline [PBS], followed by addition of trypsin-EDTA (0.25%) for 5 min at 39 °C, before adding DMEM media). Each timepoint consisted of eight biological replicates.

RNA extraction, reverse transcription and quantitative PCR

Following manufacturer protocols, RNA extraction was performed using miRNeasy Mini Kits (Qiagen, Oslo) for purification of total RNA, including miRNA, from all the cells. All eight biological replicates were pooled for RNA extraction to ensure sufficient RNA. The extracted RNA was eluted into RNase-free water and stored at −80 °C. Reverse transcription of RNA into cDNA was performed using the QIAGEN miScript II RT Kits (#218160, 218161). cDNA were diluted to a final concentration of 500 pg/µl with nuclease-free water and 3 ng of the final diluted cDNA were used for amplification (run in two technical replicates). Quantitative real-time polymerase chain reaction (qPCR) analyses were conducted using QIAGEN miScript SYBR green PCR kits (#218073). *SNORD68* was used as control gene for *mir-155* (miScript PCR controls, MS00033712, Qiagen); *28SrRNA* was used as a stable control gene for cytokine expression.

For analyzing the cDNA samples, a master mix containing SYBR green, thermostable hot-start DNA polymerase, universal primer (10 µM) was produced in the case of *mir-155* and *SNORD68*. For the analysis of all other immune genes (for *NF-κB1*, *TNFα*, *IL-8*, *IL-4* and *28 SrRNA*), gene-specific forward and reverse primers were added at a concentration of 10 µM instead. The sense and anti-sense primers used are provided in Table 1. qPCR was then done using SYBR green chemistry (as recommended by manufacturer) on the Roche Light Cycler 96. The respective temperature programs for each gene are given in Table SI-1-SI-3. For the miRNA analysis, a gga-*mir-155* miScript custom assay (MSC0003997, Qiagen) was employed.

Statistics

Data analysis was performed in Rstudio (v3.4.0) using the specialized package MCMC.qpcr (Matz et al. 2013) which presents qPCR data (i.e. C_t values) as molecular count data using generalized linear mixed models under Poisson-lognormal error. A Markov Chain Monte Carlo (MCMC) algorithm was applied to estimate effects of all random and fixed factors on the expression of every gene. Control genes *SNORD68* for *mir-155* and *28SrRNA* for *NF-κB1*, *TNFα*, *IL-8* and *IL-4* were included in the study to minimize risk of bias (Matz et al. 2013). The control gene stability for *28SrRNA* and *SNORD68* is exemplary visualized in Figure SI-1 for poly(I:C)-treated and GaHV-2-infected cells after 8 hr. Visualization of graphs was completed using the Rstudio package ggplot2 and Inkscape 0.92.

Data analysis utilized a one-way design model in which the different treatments are fitted as the single factor which is compared to the control (media only) (Waugh et al. 2018). The single response variable is the natural logarithm of transcript

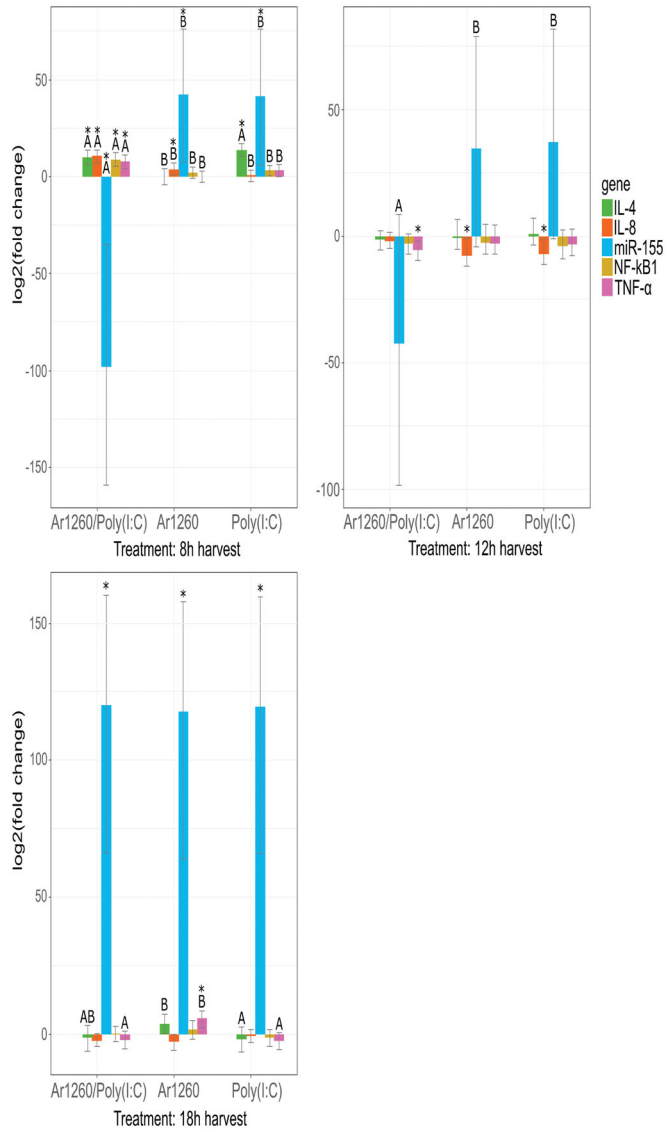

Figure 1. Gene expression rates for poly(I:C), Ar1260, and their combined treatment in CEFs – relative to in the control ("media only") – at 8, 12, and 18 h post-inoculation. SYBR green-labelled qPCR was performed with specific primers for *IL-4*, *IL-8*, *mir-155*, *NF-κB1*, and *TNFα*. Whiskers denote 95% credible intervals of the posterior distribution. Different letters indicate significant differences among treatment groups ($p < 0.05$). *Value significantly different from control ($p < 0.05$). Reactions were performed in two technical replicates.

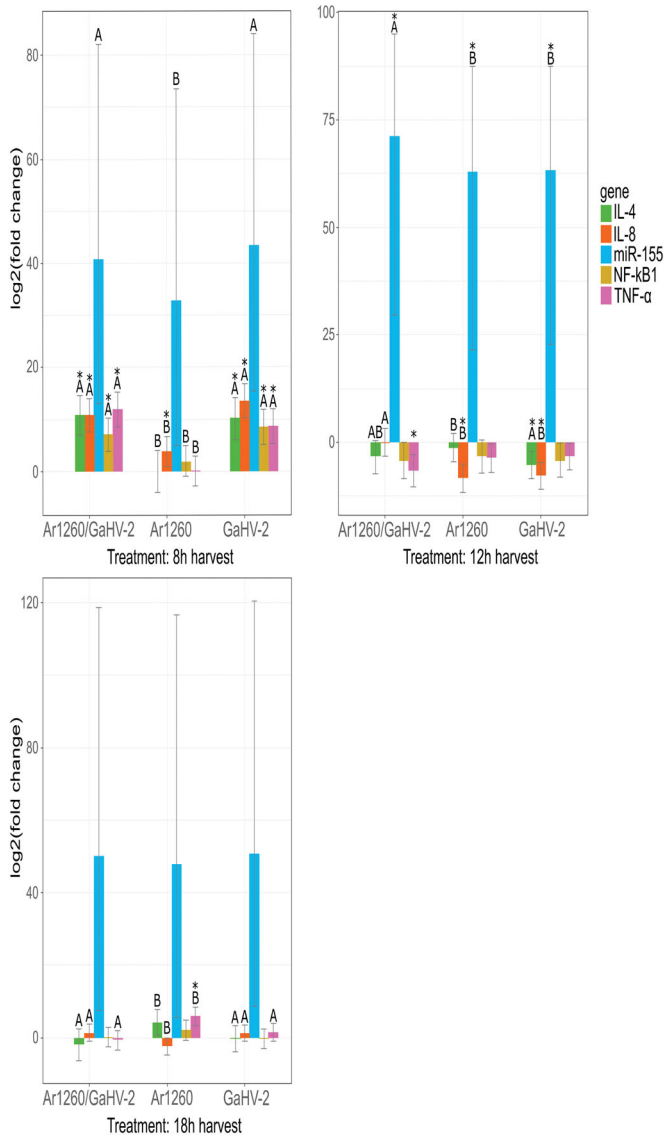

Figure 2. Gene expression rates for GaHV-2, Ar1260, and their combined treatment in CEFs – relative to in the control ("media only") – at 8, 12, and 18-h post-infection. SYBR green-labelled qPCR was performed with specific primers for *IL-4*, *IL-8*, *mir-155*, *NF-κB1*, and *TNFα*. Whiskers denote 95% credible intervals of the posterior distribution. Different letters indicate significant differences among treatment groups ($p < 0.05$). *Value significantly different from control ($p < 0.05$). Reactions were performed in two technical replicates.

counting rate. Different levels of expression between genes were explained using the explanatory variable "gene". Further on, the model was calculated with gene-specific effects for the treatments (gene:treatment), as well as random sources of variation on gene expression in, for example, the biological and technical replicates (*sample*) (Equation 1).

$$\text{Ln}\ (\text{rate}) \sim \ \text{gene} \ + \ \text{gene} : \text{treatment} \ + \ \textit{sample} \qquad (1)$$

The Markov chain Monte Carlo was run with 14,000 iterations, discarding the first 4000. The MCMC-based *p*-values and 95% credible intervals were calculated for each estimated parameter.

Estimation of statistical significance

Significant fixed effects refer to those in which the 95% credible interval (Bayesian analog to confidence interval) did not include zero. The credible interval contains the true value of the parameter within a set probability (0.95 in this case); confidence interval refers to the range that included the true parameter value in 95% of the independent re-runs of an experiment (Matz et al. 2013). The MCMC.qpcr package calculated *p*-values for all potential effects of interest and corrected for multiple testing. To calculate *p*-values, posterior distribution of a parameter of interest was assumed normally distributed for calculation of Bayesian *z*-scores (mean of posterior/SD). A standard *z*-test was also performed to derive a two-tailed *p*-value.

Results

Figure 1 presents $\log_2$-fold changes (relative to the untreated control) from the poly(I:C) or GaHV-2, Ar1260, Ar1260/poly(I:C), or Ar1260/GaHV-2 treatments at 8, 12, and 18 h. All fold-change and significant values for the treatments are given in

Supporting Tables SI-4–SI-18. Poly(I:C) or GaHV-2 alone represent responses of "non-polluted-infected" hosts to a virus. Ar1260 represents a polluted host without a virus (polluted/uninfected). Ar1260/Poly(I:C) or Ar1260/GaHV-2 represent responses of an "polluted/infected" host to a virus infection. Using this approach, one can describe how presence of multiple stressors (infection, pollution) significantly modulate natural immune responses of hosts infected with RNA (poly(I:C)) or DNA (GaHV-2) virus.

Effects of Ar1260 on expression of important immune response genes

How Ar1260 alone modulated immune responses in the absence of viral activation was evaluated first. Compared to untreated control cells, CEF treated with Ar1260 showed significant modulations across the time series. The most distinct was that *miR-155* was up-regulated at all timepoints (Figures 1 and 2; Tables SI-6, SI-11, SI-16). At 8 h, *IL-8* was significantly up-regulated (Table SI-5) but at 12 h significantly down-regulated (Table SI-10); by 18 h it had returned to status quo (Table SI-15). In comparison, *TNFα* mRNA levels were significantly up-regulated at 18 h (Table SI-18), but no earlier. Neither *IL-4* nor *NF-κB1* mRNA levels were modulated by Ar1260 at any timepoint.

Effects of Ar1260 in combination with virus analog poly (I:C) on immune response genes

By the first harvest (8 hr), poly(I:C) caused a significant up-regulation of *mir-155* and *IL-4* mRNA expression in poly(I:C) treated cells (Figure 1). Expression patterns in the Ar1260/Poly(I:C) hosts were significantly modulated from this baseline; specifically, expression of *mir-155* was significantly down-regulated (Table SI-6) while that of all other immune genes analyzed were significantly up-regulated.

In Ar1260/Poly(I:C) treated cells, expression of *mir-155* continued to be significantly down-regulated at 12 h compared to levels seen with non-polluted/poly(I:C) treated cells (Table SI-11), indicating continued suppression of a normal immune response to virus infection. However, the modulation of the other immune genes had returned to a similar expression pattern to that seen in non-polluted/poly(I:C) treated cells. By 18 h, expression patterns were largely similar between treatments, demonstrating that the majority of the effect occurred early during exposure and infection (Figure 1).

Effects of Ar1260 and DNA virus (GaHV-2) on immune response genes

After 8 h of GaHV-2 infection, in non-polluted cells the majority of immune response genes showed a significant up-regulation (Figure 2) indicating they are important in non-polluted/infected hosts for responding to this DNA virus. *miR-155* was not significantly up-regulated in non-polluted/infected vs. non-polluted/uninfected control cells (Table SI-21). There were no differences in these profiles in the polluted/infected (Ar1260/GaHV-2) cells compared to the non-polluted/infected (GaHV-2) cells at 8 h. Whereas expression profiles with these two treatments did not significantly differ, they did differ from polluted/uninfected cells, suggesting the virus may be over-riding any response to the pollutant.

By 12-h post infection, the expression profiles switched from largely immune response gene-regulated to an *mir-155*-dominated pattern (Figure 2). *mir-155* expression was significantly up-regulated by all treatments (Table SI-26), with the effect significantly greater in polluted/infected (Ar1260/GaHV-2) cells than in either non-polluted/GaHV-2-infected or polluted/uninfected cells (Table SI-26). On the other hand, *IL-4*, *IL-8*, *NF-κB1*, and *TNFα* mRNA expression tended toward down-regulation after 12 h in the Ar1260/GaHV-2 and unpolluted/GaHV-2-infected cells (Table SI-24-28). An outcome that contrasted with the observations at 8 hr. While *TNFα* was the only gene significantly down-regulated in Ar1260/GaHV-2-treated cells, the regulation did not differ significantly compared to the other treatments (Table SI-28). Further, the down-regulations of *IL-4* and *IL-8* mRNA were significant in the non-polluted/GaHV-2-infected cells (Table SI-24, SI-25).

At 18-h post infection, the expression of *mir-155* and the other immune response genes in Ar1260/GaHV-2- and GaHV-2-treated cells did not significantly differ from the non-treated control cells (Table SI-29-33). Taken together, this indicated largely that the important modulations, and the immune response, occurred early on in infection, i.e. within first 12 h.

Discussion

This study investigated effects of PCBs on important immune response genes as well as on the immune response to virus infections. This study looked at both RNA (virus analog poly(I:C)) and DNA (GaHV-2) virus examples. This study also had a large focus on *mir-155* as this has been suggested in previous work (see Waugh et al. 2018) to be a potentially under-described component of select innate immune system signaling pathway that could be modulated in response to PCBs.

Responses after treatment with Ar1260

Unlike where *mir-155* expression was seen as down-regulated after 48 h in primary CEFs exposed to Ar1250 (Waugh et al. 2018), here, 24 h exposure to Ar1260 resulted in up-regulated *mir-155* levels at all harvesting timepoints examined. The previously described disruption of *mir-155* expression was assumed to be related to an Aroclor-mediated disruption of *NF-κB* signaling. In the current study, *NF-κB1* mRNA levels were down-regulated by 36 h post-exposure (12 h post-infection) whereas there was up-regulation at all the other timepoints. As such, one could assume other Aroclor-mediated induction pathways might be a basis for the observed up-regulation. For example, Ar1260 is able to bind the aryl hydrocarbon receptor (AhR) (Safe 1994; Luthe et al. 2008; Wahlang et al. 2014) and AhR ligands are known to activate c-Jun N-terminal kinase (JNK) signaling, which might be a basis for increased induction of *mir-155* by Ar1260 (Henklova et al. 2008; Wahlang et al. 2014). In the end, it is possible that these observed differences between the present study and Waugh et al. (2018) results might be timepoint-specific, solvent-related (corn oil vs. DMSO), due to percentage chlorine (by weight in Ar1250 vs. Ar1260), or because of the use of primary CEF vs. a CEF cell line.

Interestingly, *TNFα* mRNA levels in Ar1260-treated cells showed the same regulation trend as *NF-κB1* - but the *TNFα* underwent significant up-regulation after 18 h. *TNFα* is known to be expressed upon *NF-κB* signaling; as *TNFα* itself strongly activates *NF-κB* it thus plays a central role in amplifying and extending inflammatory processes (Wallach et al. 1999). Levels of

IL-4 reflected a similar regulation trend as with *NF-κB1* and *TNFα*, suggesting similarities in induction pathways (Zamorano et al. 2001). The significant increase in *IL-8* at 32 h post-exposure (8-h post-infection) might be associated with activation of AhR, since human macrophage *IL-8* levels increase in response to AhR ligands (Vogel et al. 2005).

Responses to RNA virus analog (poly(I:C))

The demonstrated significant up-regulation of *mir-155* in non-polluted/poly(I:C)-treated cells seen here was consistent with previous studies (Hu et al. 2015; Waugh et al. 2018). Up-regulation of *mir-155* during viral infections is regarded as beneficial due to an enhanced anti-viral immunity (Tili et al. 2013; Mehta and Baltimore 2016). However, it should be noted that over-expression of *mir-155* is linked to the oncogenicity of viruses (O'Connell et al. 2009; Zhao et al. 2011). Therefore, *mir-155* levels must be tightly regulated in response to infections.

Induction of *mir-155* was previously assumed to be related to *TNFα* autocrine/paracrine signaling (O'Connell et al. 2007). However, in the current study, *TNFα* mRNA expression was not up-regulated along with miR155 in poly(I:C)-activated cells at the later timepoints evaluated (i.e., 12 and 18 h). Therefore, other mechanisms of *mir-155* induction in CEF are probably involved during the later stages, i.e. the JNK pathway through transcriptional activation of *mir-155*-encoding *bic* gene and the AP-1 complex (O'Connell et al. 2007; Tili et al. 2013). Moreover, *mir-155* targets the suppressor of cytokine signaling 1 (SOCS1) and SH2 domain-containing inositol 5′phosphatase 1 (SHIP1), thereby regulating NF-κB activity (Mann et al. 2017). However, in the current study, *NF-κB1* mRNA expression was comparable to that of *TNFα* mRNA along with *mir-155* only during the early stages (8 h) of exposure to poly(I:C).

The up-regulation of *IL-8* mRNA levels after 8 h in unpolluted/poly(I:C)-treated cells was in agreement with a previous study (Haunshi and Cheng 2014). This outcome was assumed to be related to TLR3 signaling and regarded as an important mechanism in host resistance against viral infections (Abdul-Careem et al. 2009; Haunshi and Cheng 2014). Lastly, the significant up-regulation of *IL-4* mRNA levels seen in the current study at 8 h is regarded as beneficial as measure to control inflammatory signaling (Ghiasi et al. 1999; Zamorano et al. 2001).

Taken together, these results indicated that during the early stages of an infection (i.e., up to 8 hr), *mir-155* possibly contributed to a feed-forward loop that amplified *NF-κB/TNFα* signaling whereas after 12 h regulatory mechanisms seemed to take over to down-regulate pro-inflammatory cytokine expression induced by poly(I:C). In a viral infection *in situ*, this would possibly be useful to help a host avoid developing excessive or chronic inflammation.

Effect of Ar1260 on responses to poly(I:C)

Whereas CEF seemed to react to poly(I:C) by up-regulating *mir-155* levels, the combination with Ar1260 resulted in a disruption of *mir-155*-inducing pathways after 8 and 12 h (*mir-155* was significantly down-regulated). *mir-155* deficiency in mice resulted in defective B- and T-cell responses that were linked to impaired antigen presentation and reduced antibody production (Faraoni et al. 2009). Further, a previous study demonstrated that *mir-155* deficiency in mice ultimately resulted in reduced competence to fight infections; this was associated with the role of *mir-155* as an inflammatory amplifier (Mann et al. 2017). The disruption of *mir-155* expression therefore indicates that continuous exposure to Ar1260 results in reduced host immunity and aggravated outcomes during RNA virus infections. The observed down-regulation of *mir-155* expression seen here was in agreement with findings by Waugh et al. (2018) wherein *mir-155* was significantly down-regulated compared to control and to poly(I:C)-treated cells after 24 hr. However, in contrast to that study, the present study demonstrated a down-regulation of *mir-155* only with the combined treatment. Interestingly, at 18 h after poly(I:C) treatment, the cells showed a significant up-regulation in all treatments, opposite to the previously described effects after 24 h (Waugh et al. 2018).

Except for *IL-4*, all investigated immune response genes showed an increased expression due to the Ar1260 in combination with the poly(I:C) after 8 h. This indicated that Ar1260-mediated disruption of *NF-κB* signaling was not occurring during early stages, as was seen by Waugh et al. (2018). Rather, the results here indicated that DAMPs generated at a sublethal Ar1260 concentration synergistically increased *NF-κB* signaling in the early stages. Further, sensing dsRNA via TLR3 in the case of poly(I:C) might not be sufficient to substantially activate *NF-κB* signaling. During later stages (i.e., 12 and 18 h), poly(I:C)-treated cells here showed a comparable regulation pattern of the investigated genes irrespective of Ar1260 exposure. This might be associated to the fact that *in situ* influxing immune cells - like macrophages - might be major sources of pro-inflammatory signals during later stages of infection.

Interestingly, Ar1260/poly(I:C)-treated cells displayed significant down-regulation of *TNFα* mRNA levels after 12 h. PCB mixtures have previously shown to dampen LPS-induced *TNFα* expression in murine macrophages after 24 h, which was assumed to be related to suppression of inflammatory enzyme production at the transcriptional level (Santoro et al. 2015).

Responses to infection with GaHV-2

The up-regulation of *mir-155* after GaHV-2 infection in non-polluted CEF seen here is in agreement with data from the study of Hu et al. (2016). The ability of CEF to react to GaHV-2 by inducing miRNA was previously demonstrated by sequence analysis; host miRNA represented the dominant form of miRNA induced in CEF during GaHV-2 infections (81%), whereas only 0.6% contributed to GaHV-2-encoded miRNA (Burnside and Morgan 2007). In T-cells, where GaHV-2 is known to induce malignant transformations, the regulation of host miRNA only contributed to 32% of all miRNA and GaHV-2-encoded miRNA represented 51% of the total pool (Yao et al. 2008). Of all the miRNA, *mir-155* was especially down-regulated in virus-transformed T-lymphoma cell lines; up-regulation of its ortholog miR-M4 was regarded as major determinant of GaHV-2 oncogenicity (Yao et al. 2009; Zhao et al. 2011). Thus, up-regulation of *mir-155* seems to represent an important mechanism for cells to not only fight GaHV-2 infection but also to avoid transformation events induced by GaHV-2.

All the immune response genes investigated here were up-regulated after 8 h of GaHV-2 infection and showed, except for *mir-155*, an enhanced expression compared to levels seen in unpolluted/poly(I:C)-treated cells. Interestingly, both GaHV-2 and poly(I:C) are known to significantly up-regulate *TLR3* mRNA in primary CEF after 8 h (Haunshi and Cheng 2014; Hu et al. 2016). However, additional signals provided by GaHV-2, like the production of DAMPs, might (in contrast to in poly(I:C)-treated cells) be a reason for the significant expression

of TLR3-induced defense mechanisms like the activation of *NF-κB* signaling. One study revealed that TLR3-mediated anti-viral effects in GaHV-2-infected CEF were associated with production of inflammatory cytokines (Zou et al. 2017). Therefore, modulation of *NF-κB* signaling pathways was expected to represent an important target during GaHV-2 infection. After 8 h, the CEF here were still able to induce *NF-κB1* in response to GaHV-2-infection. During later stages, a modulation of *NF-κB1* signaling might be a cause for the general down-regulation observed after 12 hr, since GaHV-2 has been shown to be able to down-regulate TLR3 protein expression in CEF (by a targeting TLR3 via miR-M4; Hu et al. [2015]).

The regulation of *TNFα* in GaHV-2-infected cells showed an expected similar regulation pattern to that for *NF-κB1*. Since *TNFα* exerts anti-viral properties against herpesviruses (Seo and Webster 2002), an up-regulation here after 8 h is expected to be a beneficial event in that it helps to inhibit GaHV-2 replication. The up-regulation of *IL-8* mRNA expression here after 8 h is in agreement with a previous study where *IL-8* was up-regulated after 8 h in GaHV-2-infected CEF. This outcome is regarded as beneficial event during GaHV-2 infection due to the chemo-static properties of *IL-8* that are assumed to be linked to resistance against GaHV-2 (Parcells et al. 2001; Haunshi and Cheng 2014). Interestingly, GaHV-2 has been shown to produce its own ortholog (v*IL-8*), which in contrast to chicken *IL-8*, attracts B- and T-cells (main hosts of GaHV-2) but fails to attract heterophils (Parcells et al. 2001; Engel et al. 2012). Thus, any active down-regulation effects induced by GaHV-2 might be a cause for the observed significant down-regulation of *IL-8* seen after 12 h. *IL-4* mRNA underwent a significant down-regulation in GaHV-2-infected cells after 12 h; this further indicated that GaHV-2 was capable of actively down-regulating expression of several key immune response genes in CEF. A down-regulation of *IL-4* is assumed to have a negative impact on the adaptive immunity due to the function of *IL-4* in the differentiation of antigen-naïve T-cells (Xing et al. 2008).

Effect of Ar1260 on responses to GaHV-2 infection

Ar1260/GaHV-2 treated cells showed an increased upregulation of *mir-155* after 8 and 12 h compared to Ar1260 polluted/uninfected cells, indicating a GaHV-2-related induction of *mir-155* during early stages. After 12 hr, *mir-155* expression in the Ar1260/GaHV-2-treated cells was significantly higher compared to that seen with both individual treatments; this demonstrated a synergism with respect to the expression of *mir-155*. Therefore, opposite to what was seen in Ar1260/poly(I:C)-treated cells, these CEF seemed to react to the combined treatment with an over-expression of *mir-155*, which has been linked to malign transformations by targeting oncogenic suppressors or anti-inflammatory signaling pathways such as SOCS1 and SHIP1 (O'Connell et al. 2009; Tili et al. 2013). The present results suggest that expression of *mir-155* can be induced by various pathways, i.e. GaHV-2 seemed to induce a different pathway that was itself enhanced by Ar1260 pre-exposure – whereas the poly(I:C)-induced pathway seemed to be disturbed in the Ar1260 pre-exposed cells. An over-expression of *mir-155* has not only been linked to chronic inflammation and cancer, but also to autoimmune disorders as well as cardio-vascular diseases (Faraoni et al. 2009). The combination of continous host exposure to persistent immunomodulating pollutants together with an infection by a DNA virus that is capable of inter-fering with highly-regulated innate immune system pathways is therefore expected to represent an important link between chronic inflammation and cancer.

All of the immune response genes investigated here were comparably up-regulated after 8 h in Ar1260/GaHV-2-treated and in unpolluted/GaHV-2-infected cells. These results indicated that GaHV-2 was the main agent involved in induction of *NF-κB*-dependent signaling after 8 h, since Ar1260 polluted/uninfected cells did not significantly up-regulate expression of any of the investigated genes. Similar observations were made in a study of perfluorooctane sulfonate (PFOS)/GaHV-2-infected CEF, wherein the combined treatment resulted in a similar expression of *NF-κB1*, *TNFα*, *IL-8*, and *IL-4* compared to unpolluted/GaHV-2-infected CEF (Castaño-Ortiz et al. 2019).

After 12 and 18 h, most of the investigated genes showed comparable levels with and without Ar1260 pre-exposure – with the exception of *IL-8* after 12 h in Ar1260/GaHV-2 treated cells, which was significantly different from levels seen in both individual treatments. This observation indicated that pre-exposure to Ar1260 may prevent GaHV-2-mediated down-regulation of some genes in CEF. Similar observations were made for *IL-4*, i.e. unpolluted/GaHV-2-infected CEF had significantly down-regulated *IL-4* levels after 12 h whereas in cells that under-went a combined exposure there was only a slight down-regulation. The results at 18 h further indicated that most cytokine regulation in GaHV-2-infected CEF took place during the early stages (i.e., up to 12 hr), since none of the investigated mRNA were significantly up- or down-regulated by this time.

Conclusions

The results of the study demonstrate how the PCB mixture Aroclor – in combination with RNA and DNA virus infections, modulates the expression of tightly-regulated innate immune system signaling pathways. Such modulations are expected to have even long-term detrimental effects for hosts including for example chronic inflammation and cancer, and might ultimately contribute to outbreaks of infectious diseases in polluted areas.

The combination of poly(I:C) and Ar1260 revealed a disruption of *mir-155* expression up to 12 h after poly(I:C) treatment, which likely poses a deleterious effect for a host due to involvement of *mir-155* in inducing early anti-viral responses. In contrast, exposure to Ar1260 resulted in increased *mir-155* expression after 12 h in GaHV-2-infected cells. Over-expression of *mir-155* is known to be detrimental to host immunity and could ultimately also potentiate cell transformations. Due to the observed differences in poly(I:C)-treated and GaHV-2-infected cells, the results lead us to conclude that GaHV-2-infected cells induce *mir-155* independent of TLR3 signaling. Further, differences between a viral analog and an actual viral infection highlight the importance of testing multiple stressors when assessing immunotoxic potentials of chemicals and adds important new evidence that *mir-155* signaling represents a sensitive pathway subject to chemical-induced (immuno)modulation.

Future studies of other early signals during viral infections (like CXCL9, CXCL10, interferons or miR-146a) in combination with expression patterns of proteins (to account for any post-transcriptional modulation) might yield further insights into the regulation of signaling pathways induced by poly(I:C) or GaHV-2 and their potential modulation by PCB mixtures.

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