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Lysis of HIV-1 Infected Autologous CD4+ Primary T cells by Interferon-alpha Activated NK cells Requires NKp46 and NKG2D

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Abstract

Objective—Autologous HIV-1 infected $CD4^+$ primary T cells ($aHIV^+CD4$) have been shown to be largely resistant to Natural Killer (NK) cell mediated lysis due to viral strategies of immune evasion. We have previously shown that a pre-activation of NK cells with Plasmacytoid Dendritic Cells can significantly augment lysis of $aHIV^+CD4$ through a mechanism dependent on Interferon-alpha (IFN-α).

Design—The goal of the present study is to identify the specific NK activating receptors involved in NK lysis of $aHIV^+CD4$ following IFN-α activation.

Methods—PBMC were incubated with $aHIV^+CD4$ to induce the secretion of endogenous levels of IFN-α and drive NK activation. We then utilized a standard chromium lysis assay to assess the degree of IFN-α activated lysis of $aHIV^+CD4$ in the presence or absence of masking antibodies to a panel of NK activating receptors and co-receptors.

Results—Direct recognition of HIV-1 infected, but not uninfected, autologous $CD4^+$ primary T cells by PBMC induced the secretion IFN-α (Median 2280 pg/ml, $p<0.001$, n=9) that, in turn, activated NK cells ($p<0.001$, n=12) and significantly increased their cytolytic potential against $aHIV^+CD4$ ($p<0.01$, n=12). The masking of NKp46 ($p<0.01$, n=8) and NKG2D ($p<0.05$, n=8), but not 2B4, NTBA, NKp30 or NKp44, significantly reduced IFN-α activated lysis of $aHIV^+CD4$.

Conclusions—Taken together, these results demonstrate that endogenous levels of IFN-α secreted by pDCs induce NK cells to lyse $aHIV^+CD4$ via the engagement of NKp46 and NKG2D.

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The authors have no financial conflicts of interest.

Author Contributions

Costin Tomescu (Completed the *in vitro* assays, co-analyzed the data, co-wrote the manuscript), *Domenico Mavilio* (Co-coordinated the study design, edited manuscript), *Luis J. Montaner* (Coordinated the study, co-analyzed the data, co-wrote the manuscript.

Keywords

AIDS; NK Cells; NKp46; NKG2D; Interferon-alpha; Cytotoxicity

Introduction

The ability of NK cells to discriminate between normal and abnormal cells involves complex interactions between inhibitory (iNKRs) and activating (aNKRs) NK cell receptors [1–5]. Under physiological conditions, the binding of iNKRs such as NKG2A and killer immunoglobulin-like receptors (KIRs) to autologous MHC-class-I (MHC-I) molecules induces negative regulatory signals that switch off NK cells [6–11]. *Heterologous* target cells expressing iNKR-mismatched MHC-I proteins exhibit a naturally increased target cell sensitivity to NK cell lysis. In contrast, normally resistant *autologous* target cells become susceptible to NK cell cytotoxicity during viral infection or tumor transformation when MHC-I proteins are down-regulated. Following the reduction of inhibitory signals, NK cells then require the engagement of aNKRs to induce the killing of susceptible target cells. Examples of aNKRs include: the NKG2D receptor that recognizes stress-induced ligands [12–15], the Fc-γIII receptor (CD16) which mediates antibody dependent cytotoxicity [16–18], activating KIRs lacking inhibitory motifs [19–21], and the Natural Cytotoxicity Receptor Family (NKp46, NKp30, NKp44) which directly recognize viral or cellular antigens [22–27]. NK cell effector functions are also modulated by co-stimulatory receptors such as 2B4 or NTBA that can synergize with other aNKRs to induce higher levels of cellular lysis [28, 29]. Likewise, cytokines such as IL-2, IL-12, IL-15, IL-21 or Interferon-alpha (IFN-α) can also augment lysis of susceptible targets cells by pre-activating NK cells [30–38].

The autologous HIV-1 infected $CD4^+$ primary T cell ($aHIV^+CD4$) NK assay system represents the most physiologically relevant *in vitro* model for measuring NK activity due to the complete match between MHC-I alleles on HIV^+CD4 target cells and iNKRs on NK cells [39–41]. However, $aHIV^+CD4$ have been shown to be largely resistant to lysis by NK cells *in vitro* due to viral strategies of immune evasion [39, 42, 43]. We have previously shown that NK cytotoxicity against $aHIV^+CD4$ can be significantly augmented by Plasmacytoid Dendritic Cell (pDC) activation of NK cells through an IFN-α dependent-mechanism [44]. We have also observed that purified pDC alone are sufficient to recognize $aHIV^+CD4$ and secrete high amounts of IFN-α that in turn can activate NK cells [45]. However, the specific receptors utilized by NK cells during IFN-α activated lysis of autologous HIV^+CD4 remains undetermined. Using a modified version of our $aHIV^+CD4$/pDC recognition system, we now investigated the specific aNKRs involved in lysis of $aHIV^+CD4$ following activation of NK cells with endogenous levels of IFN-α.

Materials and Methods

HIV-1 infection

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 healthy uninfected donors according to informed consent and Institutional Review Board approval from The

Wistar Institute. PBMCs were stimulated for 3 days with 10 μg/ml PHA-p (Sigma Aldrich, MO) and 100 IU/ml hIL-2 (PeproTech, Rocky Hill, NJ). $CD4^+$ primary T cells were isolated by positive selection using anti-CD4 magnetic beads as described by the manufacturer (Miltenyi Corporation, CA). 5×10^6 activated $CD4^+$ T cells were spinfected with 150 ng of p24 containing supernatant of the CXCR4-tropic HIV-1 isolate TYBE as previously described [44]. After 4 days of infection, we enriched HIV-1 infected cells that downregulated the CD4 receptor during infection (X>70% infectivity per donor) by removing uninfected $CD4^+$ T cells using anti-CD4 depletion magnetic beads (Miltenyi) as previously described [39].

Flow cytometry

The following antibodies were used at the recommended dilution of 0.25 μg antibody/million cells: CD3 (SK7), CD4 (SK3), CD16 (3GB), CD56 (B159), CD69 (FN50). Cell surface staining for CD69 activation was carried on $CD56^+/CD3^-$ gated NK cells with gates set upon unstimulated control cells. For intracellular staining of the HIV-1 p24 gag protein, $CD4^+$ T cells were permeabilized with the Cytofix/Cytoperm kit (BD Pharmingen) as described by the manufacturer and stained with the anti-p24 KC57 FITC antibody (Beckman Coulter, CA). Samples were collected on a LSRII Cytometer (BD) and were analyzed with FlowJo software (Tree Star Incorporated, Ashland OR).

NK chromium^{51} release cytotoxicity assay

HIV-1 infected or uninfected $CD4^+$ primary T cells were generated over a 7 day period as described above and incubated with autologous PBMC isolated from a second blood draw at a 25:1 PBMC:CD4 ratio for 18 hours as depicted in Figure 1, **panel A**. Following overnight incubation, NK cells were tested for upregulation of the CD69 activation marker by flow cytometry and IFN-α secretion into the supernatant was measured by Interferon alpha-2a ELISA (PBL Biomedical Laboratories, NJ) as described previously [44]. On the day of chromium lysis assay, a second aliquot of HIV-1 infected $CD4^+$ primary T cells was labeled with 100 μCu $Na_2{}^{51}CrO_4$ for 3 hours and incubated with autologous PBMC effector cells (containing activated NK cells as described above) at a 100:1 PBMC/CD4 ratio in a 4-hour chromium lysis assay as described previously [44]. Saturating concentrations of specific monoclonal antibodies (mAbs) were added to the PBMC cultures for masking experiments utilizing a one fourth total volume of antibody per well (50 μl in a 200 μl total volume). The following masking antibodies (mAbs) kindly provided by Prof. Alessandro Moretta were used for the study: BAT221 (IgG1, anti NKG2D), BAB-281 (IgG1, anti-NKp46), F252 (IgM, anti NKp30), KS38 (IgM, anti-NKp44), KL247 (IgM, anti-NKp46), CO54 (IgM, anti-2B4), ON56 (IgG2a, anti-NTBA), and FS280 (IgG2a, anti-CD56). The appropriate IgG1 or IgM blocking antibody for NKp46 was chosen for blocking experiments when compared to NKG2D (IgG1) and NKp30/NKp44 (IgM) while anti-CD56 was used as a control antibody.

Statistical analysis

All graphic presentations and statistical analysis were performed with Prism software (GraphPad Software, La Jolla, CA). In individual representative experiments, error bars

depict the standard deviation. In composite graphs of multiple experiments, cross bars represent the median. Statistical analysis of three groups was carried out using a Friedman matched pair, non-parametric ANOVA test with a post-hoc Dunn analysis. p-values were two-sided with alphas noted in figures by asterisks as follows: $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***).

Results

Direct Recognition of aHIV$^+$CD4 induces the secretion of IFN-α, that in turn leads to NK cell activation and triggers cytotoxicity against aHIV$^+$CD4 through NKp46 and NKG2D

In order to investigate the specific NK activating receptors involved in IFN-α activated NK lysis of HIV-1 infected autologous CD4$^+$ primary T cells (aHIV$^+$CD4), we utilized a modified version of our previously published autologous experimental approach [45]. As shown in Figure 1, this model system involves the incubation of PBMC for 18 hours with aHIV$^+$CD4 to induce the secretion of IFN-α (Median 2280 pg/ml, $p<0.001$, n=9) that in turn stimulates NK activation ($p<0.001$, n=12) and significantly increases the NK cytolytic potential against aHIV$^+$CD4 ($p<0.01$, n=12). We have previously determined that depletion of pDCs from PBMC prior to incubation with aHIV$^+$CD4 abrogated the secretion of IFN-α and NK cell activation [45]. We have also shown that depletion of NK cells from IFN-α stimulated PBMCs reduced lysis of aHIV$^+$CD4 to undetectable levels and that lysis of aHIV$^+$CD4 can be recapitulated with purified NK cells incubated solely with IFN-α [44]. We next performed masking experiments with a panel of monoclonal antibodies (mAbs) specific for different NK activating receptors in order to identify the NK cell receptor pathways regulating IFN-α activated lysis of aHIV$^+$CD4. As shown in two representative experiments (Figure 2A and B) and across multiple donors (Figure 2C), the masking of either NKp46 or NKG2D significantly reduced the IFN-α activated lysis of aHIV$^+$CD4 ($p<0.01$ *and* $p<0.05$, n=8). In contrast, the blocking of the co-stimulatory receptors NTBA and 2B4 (Figure 2A) or natural cytotoxicity receptors NKp30 and NKp44 (Figure 2B) did not alter IFN-α activated lysis of aHIV$^+$CD4. Taken together, these results demonstrate that endogenous levels of IFN-α secreted by pDCs in response to HIV-1 infected target cells induce NK cells to lyse aHIV$^+$CD4 via the engagement of NKp46 and NKG2D.

Discussion

Utilizing a physiologically relevant model system for measuring NK lysis, we demonstrate here that endogenous levels of IFN-α secreted by pDCs induce NK cells to lyse aHIV$^+$CD4 via the engagement of NKp46 and NKG2D (but not 2B4, NTBA, NKp30 or NKp44). Our results showing a key role of NKG2D in triggering the NK cell-mediated clearance of aHIV$^+$CD4 are in line with previous studies reporting the up-regulation of NKG2D ligands on the surface of HIV-1 infected cells [40, 41]. We now extend this finding by demonstrating that the NKp46 receptor is also required for lysis of aHIV$^+$CD4 following IFN-α activation. As both cellular and viral antigens can act as ligands for the NKp46 receptor, an exploratory proteomic approach may be needed to discover the ligand induced on aHIV$^+$CD4 in future studies.

Of note, previous studies have also shown a role for NKp44 in NK recognition of HIV-1 infected target cells from HIV-1 infected viremic subjects [27]. In our experimental approach, we could not detect any contribution of NKp44 in the clearance of aHIV$^+$CD4 following IFN-α activation. This finding is not unexpected as NKp44 is not expressed on resting NK cells [4, 16, 23, 27, 29, 41, 46, 47]. Rather, NKp44 is up-regulated during chronic HIV-1 viral infection or following stimulation of NK cells with certain cytokines such as IL-2 (but not IFN-α) [25, 27, 29, 46, 48]. Similarly, it has been previously reported that the blocking of NKG2D, but not NKp46, reduces NK lysis of aHIV$^+$CD4 [41]. The discrepancy between this study and our results may be related to the absence of IFN-α stimulation and testing lysis of whole NK populations versus selected NK subsets as effectors. Previously, Ward et al. utilized depleted NK cell cultures where NK cells expressing inhibitory receptors were removed to stimulate NK lysis [41]. Here, we utilized endogenous IFN-α activation of total NK cell populations to overcome the immune evasion mechanisms of HIV-1. It remains to be determined if IFN-α selectively activates lysis of aHIV$^+$CD4 by NK cells expressing inhibitory receptors that have been licensed to kill during ontogeny. The implications of our data to HIV-1 infected subjects pertain to retained NK function during viral control, as prolonged viremia is associated with NK dysfunction. Specifically, during ART immune reconstitution we would expect a recovery of IFN-α-stimulated NK lytic activity against HIV-1 infected autologous CD4$^+$ primary T cells by the same mechanism as described here for NKp46 and NKG2D (and potentially NKp44). Future longitudinal studies addressing ART, the degree of CD4 reconstitution and NK/pDC functional recovery will be needed to determine the timeline to NK cell responses against autologous infected targets following ART suppression.

Taken together, our results demonstrate that pDC driven NK activation through IFN-α stimulates lysis of aHIV$^+$CD4 through a mechanism dependent on NKp46 and NKG2D. Having established the anti-HIV activity of IFN-α2 as an immunotherapy in antiretroviral therapy suppressed subjects [49], and a role for pDC frequency and control of viral replication [50, 51], our results here further highlight innate immunity and pDC/NK crosstalk in control over HIV-1. Conversely, the reduction in pDC function and NKp46 receptor expression during viremia [46, 52–54] suggests that a loss of IFN-α activated NK recognition of HIV-1 infected target cell through NKp46 may contribute to higher HIV-1 replication and disease progression.

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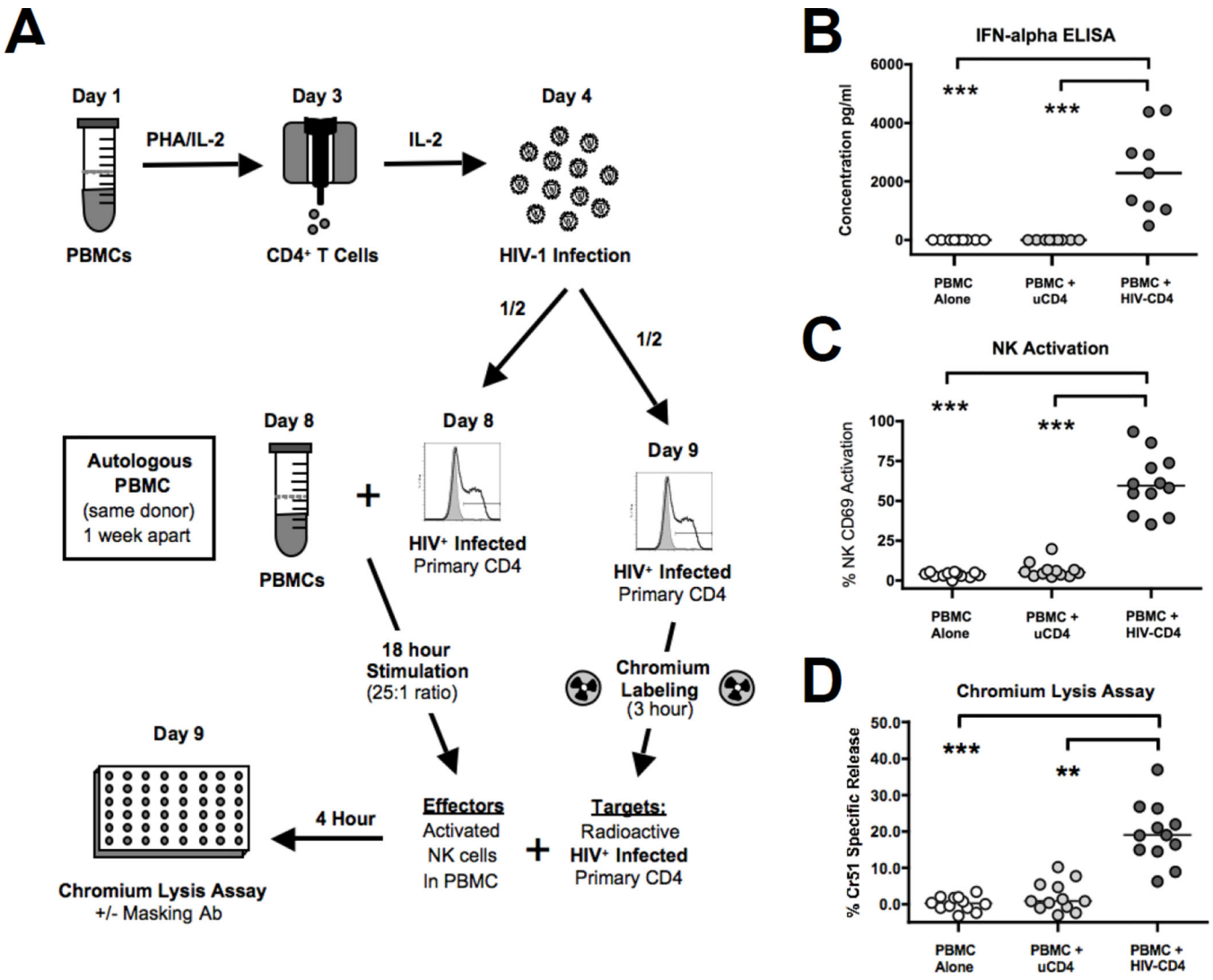

Figure 1. Endogenous IFN-α activation of NK cells stimulates lysis of HIV-1 infected autologous CD4 primary T cells (aHIV+CD4)

A) Experimental approach undertaken to determine the ability of IFN-α activated NK cells to lyse aHIV^{+}CD4 either in the presence or in the absence of masking antibodies against different NK activating receptors. PBMCs were incubated for 18 hours with HIV-1 infected or uninfected autologous CD4^{+} primary T cells at a 25:1 PBMC/CD4 ratio and then re-incubated with another aliquot of the same chromium labeled aHIV^{+}CD4 at a 100:1 E:T ratio in a standard 4 hour lytic assay. **B)** Summary graph showing the level of IFN-α (pg/ml) secreted by PBMCs following 18 hour incubation with either HIV-1 infected or uninfected autologous CD4^{+} primary T cells. Data represents 9 donors tested. **C)** Summary graph showing the surface expression of the activation marker CD69 on CD3^{-}/CD56^{+} NK cells within total PBMCs following the incubation with either HIV-1 infected or uninfected CD4^{+} autologous T cells. Data represents 12 donors tested. **D)** Summary graph showing the degree of Cr^{51} specific release as indicative of target cell lysis in PBMC incubated for 18 hours with autologous HIV-1 infected or uninfected CD4^{+} primary T cells at a 25:1 PBMC/CD4 ratio and then re-incubated with another aliquot of the same chromium labeled aHIV^{+}CD4 at

a 100:1 E:T ratio. Data represents 12 donors tested. For graphs B-D, the statistical analyses were performed by using a paired, non-parametric Friedman ANOVA with a Dunn post-test. In all cases, significant results have two-sided p values of $p<0.05$, $p<0.01$, $p<0.001$ denoted with a single, double or triple asterisk in graphs, respectively.

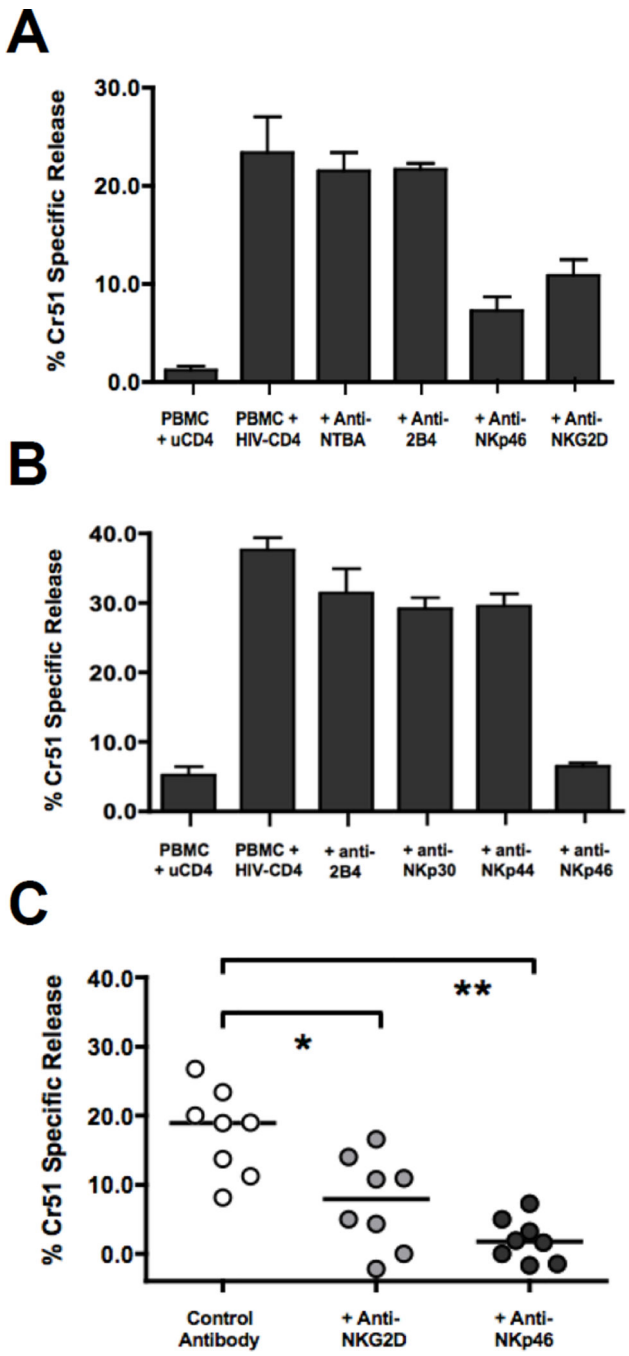

Figure 2. IFN-α activated lysis of aHIV+CD4 is blocked with masking antibodies specific for NKp46 and NKG2D

A–B) Two representative experiments showing the degree of IFN-α activated NK lysis of HIV-1 infected $CD4^+$ primary T cells (as measured by Cr^{51} specific release) in the presence or absence of masking antibodies against various NK activating receptors. Autologous PBMC were incubated for 18 hours with uninfected (first bar) or HIV-1 infected (next 5 bars) $CD4^+$ primary T cells at a 25:1 PBMC/CD4 ratio and then re-incubated with another aliquot of chromium labeled HIV-1 infected $CD4^+$ primary T cells at 100:1 E:T ratio in a four hour chromium lysis assay with masking antibodies against **A)** NTBA, 2B4, NKG2D,

NKp46 or **B)** 2B4, NKp30, NKp44, NKp46. **C)** Summary graph showing in the above-mentioned experimental approach in a subset of 8 donors where IFN-α activated NK lysis of HIV-1 infected $CD4^+$ primary T cells was measured in the presence of masking antibodies against NKG2D or NKp46 in the same experiment. The statistical analyses were performed by using a paired, non-parametric Friedman ANOVA with a Dunn post-test. In all cases, significant results have two-sided p values of $p<0.05$, $p<0.01$, $p<0.001$ denoted with a single, double or triple asterisk in graphs, respectively.