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SHORT COMMUNICATION

Antiviral activity of human Vδ2 T-cells against WNV includes both cytolytic and non-cytolytic mechanisms

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SUMMARY

West Nile virus (WNV) causes a severe central nervous system infection in humans, primarily in the elderly and immunocompromised subjects. Human γδ T-cells play a critical role in the immune response against viruses, and studies of WNV meningoencephalitis in laboratory mice described a role of γδ T-cells in the protective immune response. Aim of this study was to analyze the cytolytic and non-cytolytic antiviral activity of human Vδ2 T-cells against WNV replication.

The anti-WNV activity of soluble factor released by zoledronic acid (ZA)-activated Vδ2 T-cell lines and the cytotoxic capability of Vδ2 T-cell lines against WNV-infected cells were tested in vitro. The activation of Vδ2 T-cell lines was able to inhibit WNV replication through the release of soluble factors. IFN-γ is massively released by activated Vδ2 T-cell lines and is involved in the anti-WNV activity. Moreover, the Vδ2 T-cell lines can efficiently kill WNV-infected cells possibly through perforin-mediated mechanism.

Altogether, our results provide insight into the effector functions of human Vδ2 T-cells against WNV. The possibility to target these cells by ZA, a commercially available drug used in humans, could potentially offer a new immunotherapeutic strategy for WNV infection.

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West Nile virus (WNV) is a re-emerging pathogen responsible for fatal outbreaks of meningoencephalitis in humans. This plus-sense, single-stranded neurotropic flavivirus has been representing a public health concern in North America for more than a decade (Campbell *et al.,* 2002; Kramer *et al.,* 2008). Infections in humans mainly result from mosquito bites, but may also be acquired through blood transfusion, organ transplantation and breast feeding (2002a; 2002b; Campbell *et al.,* 2002; Charatan, 2002). Although human infection is usually asymptomatic, life-threatening neurological disease including encephalitis can ensue, particularly in the elderly and in immunocompromised hosts (Chowers *et al.,* 2001; Nash *et al.,* 2001). At present, there is no specific therapeutic agent available for either treatment or approved human vaccine for prevention.

The analysis of WNV pathogenesis and host immune response has mainly been performed using the mouse model of WNV infection (Beasley *et al.,* 2002; Kramer *et al.,* 2001). In this system, both innate and adaptive immune systems contribute to WNV infection control (Diamond *et al.,* 2003a). In fact, B cells and specific antibodies are critical in controlling viral dissemination (Diamond *et al.,* 2003b; Roehrig *et al.,* 2001), while αβ T-cells provide long-lasting protective immunity and contribute to host survival following WNV infection (Sitati *et al.,* 2006). Among innate immune cells, γδ T-cells provide a rapid response during WNV infection in mice, limiting the viral load and protecting the host from lethal encephalitis (Wang, 2011; Wang *et al.,* 2013). Murine γδ T-cells expand quickly in response to WNV infection, produce significant amounts of IFN-γ (Wang *et al.,* 2003) and promote the maturation of dendritic cells, resulting in an improved CD4 T-cell response (Fang *et al.,* 2010). Moreover, γδ T-cell-deficient mice have a reduced CD8+ T-cell memory response and are more susceptible to secondary WNV infection, suggesting a role of murine γδ T-cells in linkage of innate immunity to adaptive immune responses (Wang *et al.,* 2006). Although the murine model represents an effective experimental model to investigate WNV pathogenesis and host immunity, γδ T-cell subsets show substantial differences between mice and humans. In humans, γδ T-cells account for approximately 1%-5% of circulating T-cells, and most of them bear the Vγ9Vδ2 T-cell receptor (TCR) (Born *et al.,* 2006; Carding *et al.,* 2002). Specifically, proliferative, cytotoxic, and cytokine responses of the human Vδ2 T cell subset are induced by both nonpeptidic antigens, and nitrogen-containing bisphosphonates (N-BPs), such as zoledronic acid (ZA) (Dieli *et al.,* 2003; Hayday, 2000). Vδ2 T-cells can be easily activated *in vivo* by N-BP, and have been proposed as a target for innovative approaches to the immunotherapy of viral infections (Deniger *et al.,* 2014; Poccia *et al.,* 2005b). Notably, human Vδ2 T-cells are known to exert broad antiviral activities against different viruses through both cytolytic and non-cytolytic mechanisms (Agrati *et al.,* 2006a; Agrati *et al.,* 2006b; Poccia *et al.,* 1999; 2005a;

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2006). To date, no data are available on human γδ T-cell antiviral activities against WNV replication.

In order to assess the anti-WNV activity of human Vδ2 T-cells, Vδ2 T-cell lines were obtained *in vitro* from healthy donors (HD). In particular, Vδ2 T cell lines (purity >90%) were generated by stimulating peripheral blood mononuclear cells (PBMC) from HD with ZA (2 μM mmol/L) and IL-2 (100 U/mL) for 12 days as previously described (Agrati *et al.,* 2006b). The Vδ2 T-cell lines were then re-stimulated or not with ZA for 24 h, and the soluble factors (SF) released by unstimulated (SF-ctrl) or by ZA-stimulated (SF-ZA) Vδ2 T cell lines were diluted 1:2 and added 24 well plates containing Vero-E6 cell culture monolayer (10^5 cells/well). After overnight incubation, the cultures were infected with WNV at multiplicity of infection (MOI) of 1 tissue culture infection dose $(TCID)_{50}$/cell; after 1 h absorption period, the virus inoculum was removed and replaced by fresh medium; virus yield in culture supernatants was determined after overnight incubation, by back-titrating viral infectivity on 96 well Vero E6 culture plates by limiting dilution assay. Results showed that SF-ZA obtained from 93% of HD were able to inhibit WNV replication (*Figure 1A*, virus field reduction >1,5 log $TCID_{50}$ in 13/14 of independent experiments). When comparing the extent of the WNV yield reduction observed with SF-ZA and SF-ctrl, a lower albeit appreciable reduction was observed with SF-ctrl (SF-ctrl: 1.1±0.23 log $TCID_{50}$ *vs* SF-ZA: 2.6±0.4 log $TCID_{50}$, p<0.005, *Figure 1B*), suggesting that Vδ2 T-cell lines release anti-WNV factor(s). Data from our previous studies indicated a major role of IFN-γ in mediating the non-cytolytic antiviral activity of Vδ2 T-cells against several viruses (reviewed in Poccia *et al.,* 2005a), such as SARS-Cov (Poccia et al., 2006), orthopoxvirus (Agrati *et al.,* 2006b), HIV (Poccia *et al.,* 1999) and HCV (Agrati *et al.,* 2006a). Thus, in order to verify the IFN-γ involvement in WNV antiviral activity, IFN-γ was quantified in SF-ctrl and in SF-ZA and cytokine neutralization experiments were performed. In particular, IFN-γ quantification was performed using an ELISA assay (Biocompare, San Francisco, CA, USA), while the neutralization assay were

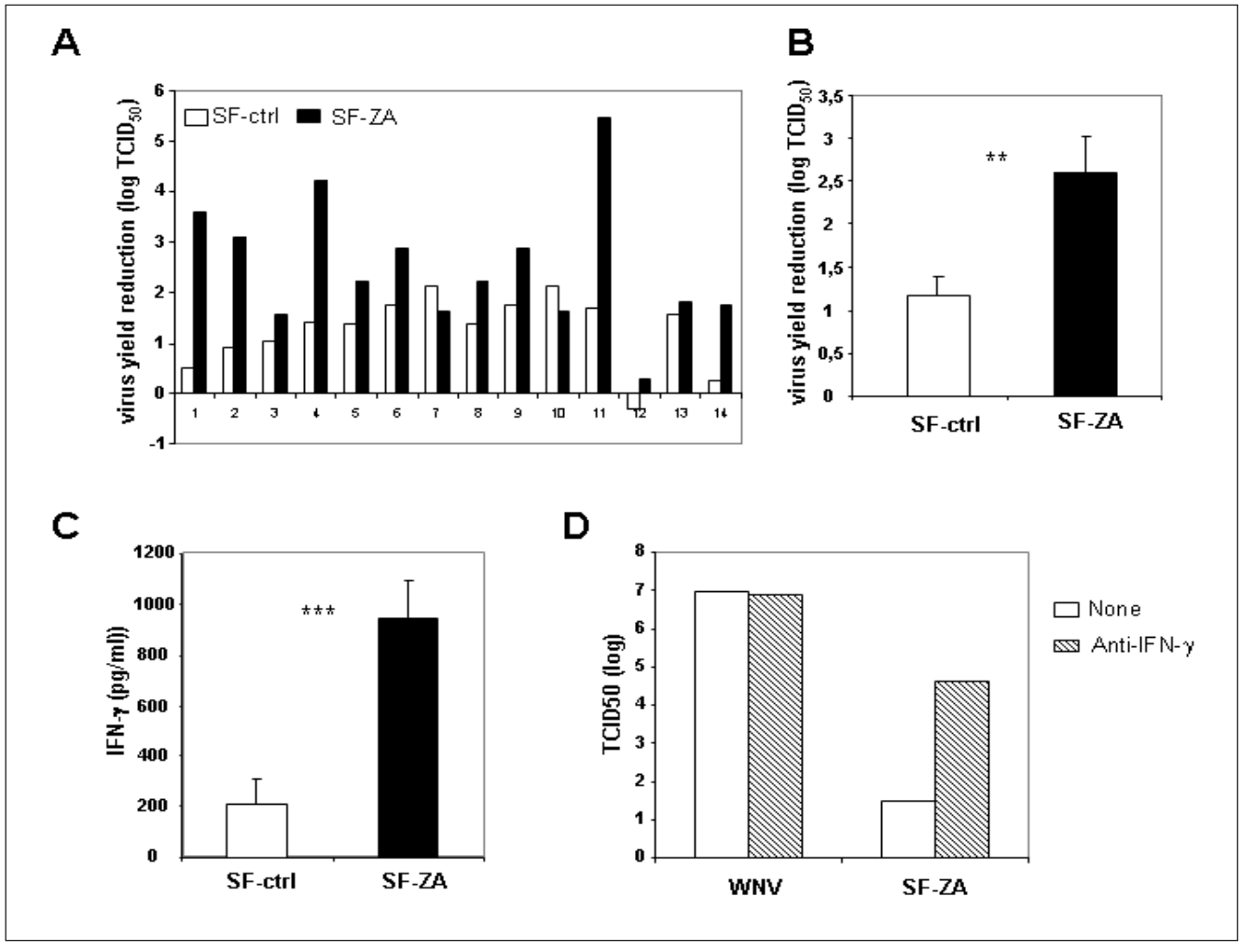

Figure 1 - *Vδ2-T cell lines efficiently inhibit WNV replication.*
*Inhibition of WNV replication by soluble factors (SF) released by unstimulated (SF-ctrl) or by ZA-stimulated (SF-ZA) Vδ2 T-cell lines. A: Individual results obtained from independent experiments with cells from 14 healthy donors are shown. Results are expressed as virus yield (Log TCID50) reduction in Vero E6 WNV-infected cultures. B: Mean ± SE over the results shown in A; **p<0.005. C: IFN-γ quantification in SF-ctrl and in SF-ZA by ELISA assay. Results are expressed as pg/ml; **p<0.0001. D: IFN-γ neutralizing antibody partially abolishes WNV inhibition exerted by SF-ZA. The at least partial abrogation of SF-ZA-driven WNV inhibition was consistently observed in 3 independent experiments. Due to considerable variability of individual results, a representative experiment is shown. Statistical analysis was performed by using Mann-Whitney test.*

performed using a rabbit anti–interferon (IFN)-γ polyclonal antibody preparation (original titer, 50,000 neutralization units/mL) at a concentration of 10,000 neutralization units/mL, as in previous experiments with other viruses (Agrati *et al.,* 2006a; Poccia *et al.,* 2006). As shown in *Figure 1C*, a significantly higher amount of IFN-γ was observed in SF-ZA when compared to SF-ctrl (944.6±149 pg/ml *vs* 211.1±99, p<0.0001). Blocking experiments showed that the anti-WNV activity of the SF-ZA was significantly, although not completely, abolished by the addition of IFN-γ neutralizing antibody (*Figure 1D*), suggesting that IFN-γ is, at least partially, responsible for Vδ2-mediated inhibition of WNV replication. This finding is not unexpected, since IFN-γ is known to participate in WNV control. In mice that are deficient in either IFN-γ or IFN-γ receptor, an increased viral burden in peripheral tissues and a rapid spread to the CNS and early death was observed (Shrestha *et al.,* 2006b). Moreover, the IFN-γ produced by murine γδ T-cells limits early virus dissemination in mice (Shrestha *et al.,* 2006b; Wang *et al.,* 2013).

We then explored the cytolytic capability of Vδ2 T-cells against WNV-infected cells. Because the functional activation of Vδ2 T-cells requires species-specific interaction with target cells (Kato *et al.,* 2003), Vero-E6 cells of simian origin could not be used in the experiments with human Vδ2 T-cells. Therefore, in these experiments we used A549 cells, a human adenocarcinoma alveolar basal epithelial cell line, infected with WNV at MOI 1. Specifically, Vδ2 T-cell lines were co-cultured with mock- (*Figure 2A*, dotted lines) and WNV-infected A549 cells (*Figure 2A*, continuous lines) at 1:1 or 5:1 Effector/Target (E/T) ratio. After 24 and 48 h, the percentage of killed cells was determined by propidium iodide (PI) labelling (Annexin V/Fitc Kit, Bender Med Systems, CA, USA) and flow cytometry (*Figure 2A*). Results showed that Vδ2 T-cells exerted a dose- and time-dependent cytotoxic activity against WNV-infected cells. A similar cytotoxic capability of Vδ2 T-cells was also described against influenza, SARS-CoV-infected cells (Li *et al.,* 2013; Poccia *et al.,* 2006). The cytotoxicity of Vδ2 T-cells can be induced by several pathways, involving T cell receptor (TCR) and/or natural killer (NK) receptors, and can be mediated by the secretion of different mediators such as perforin (Cimini *et al.,* 2011; Nedellec *et al.,* 2010). In order to evaluate the role of perforin release in Vδ2-mediated killing of WNV-infected cells, Vδ2 T-cell lines were co-cultured with mock-infected (*Figure 2B*, white bars) and WNV-infected A549 cells (*Figure 2B*, grey bars) at two different E/T ratio (1:1 and 5:1); after 48 h, a quantitative analysis of perforin released in the supernatants was performed by using the human Perforin Elisa Kit (Abcam, UK). A significant increase in perforin release was observed in WNV-infected co-cultures at E/T 1:1 ratio (Mock: 166.7±77.1 pg/ml vs WNV: 299.3±88 pg/ml, p<0.05) and to a much higher extent at 5:1 E/T ratio (Mock: 988.7±493.7pg/ml *vs* WNV: 1341±485.4 pg/ml, p<0.05), suggesting a possible involvement of perforin in

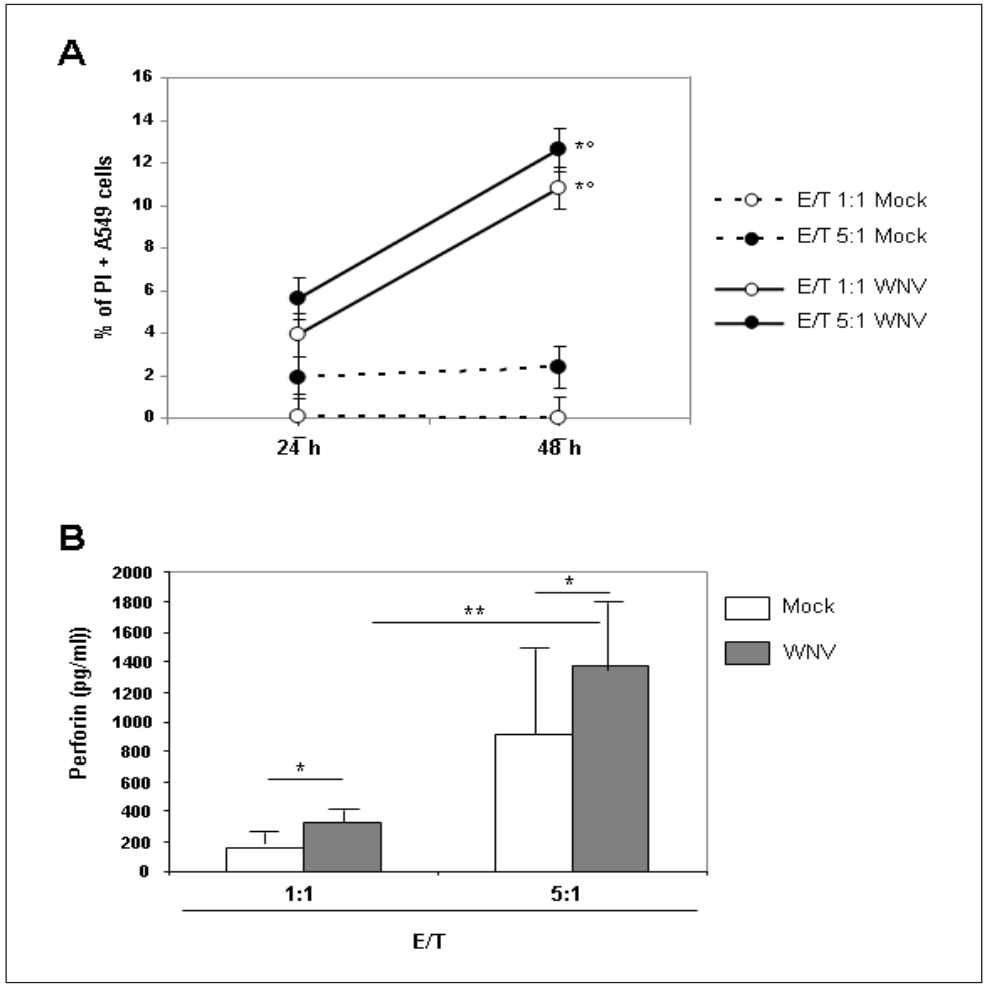

Figure 2 - *Vδ2-T cell lines efficiently kill WNV-infected cells.*
*A: The cytotoxic activity of Vδ2 T-cell lines against mock-infected (dotted lines) or WNV-infected (solid lines) A549 cells was analysed by monitoring the frequency of propidium iodide (PI)-positive cells after 24 and 48 of co-culture at 1:1 (white circles) and at 5:1 (black circles) E/T ratio. Mean results from 4 independent experiments are shown. *Comparison between Mock - and WNV -infected cells p<0.001; °comparison between 24 and 48 h p<0.005. Statistical analysis was performed using Mann-Whitney test. B: Perforin released in the supernatants of co-cultures of Vδ2 T-cell lines with mock- (white bars) or WNV-infected (grey bars) A549 cells (E/T 1:1 and 5:1). Results from 4 independent experiments are shown. *p<0.05; **p<0.005. Statistical analysis was performed using Mann-Whitney test.*

the Vδ2-mediated killing of WNV-infected cells, as previously shown for CD8 T-cells (Nedellec *et al.*, 2010; Shrestha *et al.,* 2006a). Further analyses are mandatory in order to elucidate other anti-WNV molecules in SF and to identify the molecular mechanism/s involved in Vδ2 mediated killing of WNV-infected cells.

Altogether, our results provide insight into the effector functions of human Vδ2 T-cells against WNV. The possibility to target these cells by ZA, a commercially available drugs used in humans, could potentially offer a new immunotherapeutic strategy for WNV infection.

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