Carfilzomib Impairs the Innate Antiviral Immune Response and promotes cytotoxic T-cell Expansion in Oncolytic Virus Treated Multiple Myeloma Patients

Ada Dona'[1,2§], Domenico Viola[1,2§], Enrico Caserta[1,2], Francesca Besi[3], Bharath Mulakala[1,2], James F Sanchez[1], Guido Marcucci[4], Jonathan J Keats[5], Amrita Krishnan[1], Matt Coffey[6], Douglas W. Sborov[7], Gerard Nuovo[8], Craig C Hofmeister[9*], and Flavia Pichiorri[1,2*]

[1]Judy and Bernard Briskin Center for Multiple Myeloma Research, City of Hope, Duarte, CA; [2]Department of Hematologic Malignancies Translational Science, Beckman Research Institute, City of Hope, Duarte, CA; [3]Department of Immunology, IRCSS Bambino Gesù Children's Hospital, Rome, Italy; [4]Department of Hematologic Malignancies Translational Science, Gehr Family Center for Leukemia Research, Beckman Research Institute., City of Hope, Duarte, CA; [5]Translational Genomics Research Institute, Phoenix, AZ; [6]Oncolytics Biotech, Inc., Calgary, Canada; [7]Division of Hematology, Department of Internal Medicine, University of Utah, Salt Lake City, UT; [8]Phylogeny Medical Laboratory, Powell, OH; [9]Winship Cancer Institute /Department of Hematology and Medical Oncology, Emory University School of Medicine, Atlanta, GA

Introduction

Pelareorep is an infusible form of human **Reovirus** (RV) Serotype 3 – Dearing Strain, a naturally occurring, ubiquitous, non-enveloped double-stranded RNA virus. Our single-agent phase 1 RV trial in relapsed multiple myeloma (MM) showed it selectively infected MM cells but not the bone marrow (BM) stroma. However, apoptosis of cancer cells was not observed (PMID: 25294913).
Our ongoing phase 1 trial, which combines the proteasome inhibitor carfilzomib with RV, has demonstrated RV infection, apoptosis, and clinical responses (NCT02101944) and recent published data have shown that in a mouse model PI resistant MM cells still respond to RV/BTZ combination treatment in terms of decreased tumor burden and improved overall survival (PMID: 30850386)
Here we further investigated the molecular mechanisms behind the beneficial effect of a PI (carfilzomib) in this setting.

Methods

- **Flow Cytometry for Reovirus capsid detection**—PBMCs or MM cells were fixed with 1% of formalin then stained with Purified Antibody (Protein G) Reovirus T3D for 30 min at room temp and incubated with an anti-goat IgG PE conjugated as a secondary antibody.
- **Phagocytosis assay**—Total PBMC isolated from 3 different HDs were treated over-night with RV (5MOI). After incubation CD14+ cells were isolated (Miltenyi immunomagnetic beads) and co-cultured (8:1) with Gfp+MM.1S (target cells) for 24h. Flow cytometry analysis using Gfp+ gaiting was used to assess phagocytic activity of the monocytes against the target cells.
- **PBMCs obtained from two patients enrolled on NCI 9603 (NCT 02101944)** were used for assessing immunological changes upon RV+CFZ treatment at different time points after RV infusion. Flow cytometry analysis was used to assess CD14+, CD8+ and CD4+ frequency in total PBMCs.

The combination CFZ plus RV is not able to induce a synergistic effect on Apoptosis of MM cell lines, *in vitro*

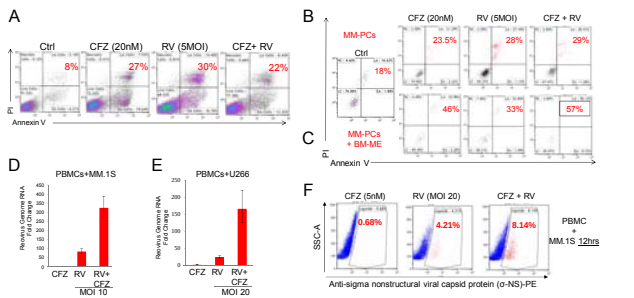

Fig. 1 PI enhances Reo infection of MM cells in the BM-ME. A) MM1.S cells were seeded and treated with CFZ (20nM) and RV(MOI 5), alone or in combination, for 24hrs and then stained with Annexin V-FITC to identify apoptotic cells (Annexin V +) and analyzed by flow cytometric analysis; **B)** and **C)** Primary CD138+ MM-PCs were treated with CFZ and RV for 24hrs, alone **(B)** or in co-culture **(C)** with the CD138- BM-ME of the same patient then labelled with Annexin V-FITC and analyzed by Flow Cytometry **D)** PBMCs from a healthy donor were co-cultured with MM.1S **(D)** and U266 **(E)** MM cells and treated with RV and CFZ (20nM), alone or in combination, for 12h to assess the Reovirus Genome Expression by q-RT-PCR; **F)** PBMCs isolated from an independent healthy donor, were treated for 4h with CFZ(20nM) and infected with RV (5MOI) then washed out and co-cultured with MM1.S cells for 12h. The membrane of the cells was fixed with 1% of formalin then stained with Purified Antibody (Protein G) Reovirus T3D for 30 min at room temp and then incubated with an anti-goat IgG PE conjugated as a secondary antibody. After 30 min of incubation the cells were washed and analyzed by flow cytometry.

CFZ inhibits anti-viral Immune Response

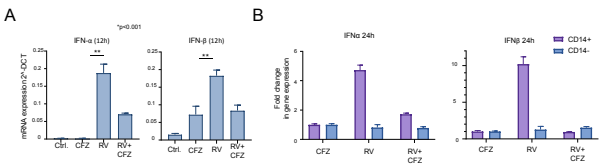

Fig.2 A-C) PBMCs, CD14+ and CD14- cells, isolated from HDs, were treated for 4h with CFZ (2.5nM) and infected with Reo (MOI 5) then washed for 12h. q-RT-PCR to detect cytokines mRNA expression (IFN-α and IFN-β), showing a significant decrease in INF-α and INF-β induction upon the combination between RV and PI. An effect that was not seen in the CD14 negative fraction of the PBMCs.

CFZ enhances RV replication in Monocytes

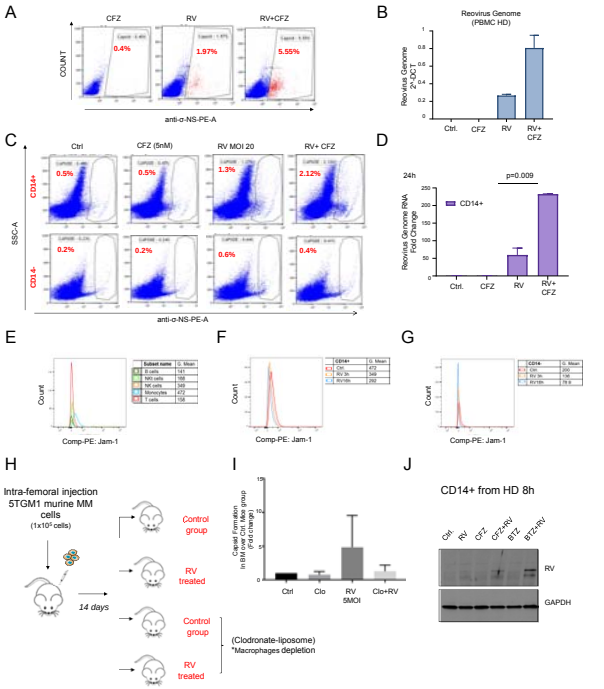

Fig.3 A-C) viral capsid detection by flow cytometry in the total PBMCs **(A)**, CD14+ and CD14neg. **(C)** fractions, isolated from HDs, treated with PBS (Ctrl) or with CFZ (5nM) for 4hrs +/- Reo for 24h, and **B-D)** q-RT-PCR to detect Reovirus genome expression normalized for GAPDH in the total PBMC (B) and Monocytes fraction (D) showing that the combined treatment (PI+RV) increases both the viral genome and the protein capsid formation; **E-G)** Overlay histogram showing JAM-1 basal level expression in different immune-subsets, and in CD14+ **(F)** and CD14- fractions **(G)** after 3-16hrs of infection with RV; **H)** schematic representation of mice experiment: 18 immune competent myeloma mice (C57BL/KaLwRij) were injected Intra-femorally with1x105 5TGM1 murine myeloma cells and treated with 150 mg/kg clodronate-liposome for monocytes-macrophages depletion or intravenously injected with RV (5 × 108 TCID50) for 24hrs; **I)** Bar graph showing bone marrow Reovirus Capsid formation of mice in treatment analyzed by flow cytometry. **J)** Western blot analysis of CD14+ cells isolated from HDs and treated with 2.5nM of CFZ or BTZ alone or in combination with RV 5MOI for 24 hrs showing higher viral σ-NS capsid protein upon RV infection in combination with PI and Bay11. Data are expressed as the mean ± SEM (n=3), normalized compare to control β-Actin;

RV induces monocytes activation against myeloma cells

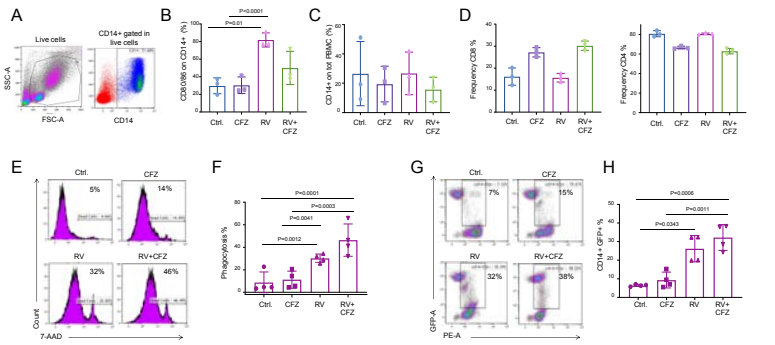

Fig.4 A) Dot plot showing representative gating strategy for identification of Monocytes cells gated in total PBMC live cells isolated from 3 different HDs; **B)** Bar graph showing up-regulation (p<0.001compared to the control) of CD80/86 in the monocytic fraction of PBMC isolated from n=3 HDs, treated for 24hrs with RV or CFZ alone or in combination; **C)** Bar graph representing the mean ±SD of CD14+ frequncy in total PBMC; **D)** Flow analysis of CD4+ and CD8+ frequency showing no differences between the RV treated samples and the control. Each experiment was repeated using three HDs in triplicate; **E-F)** Flow cytometry based phagocytosis assay on Total PBMC isolated from 3 different HDs treated over night with RV 5MOI and CFZ 2.5nM alone or in combination. After incubation CD14+ cells were isolated and co-cultured (8:1) with Gfp+MM.1S (target cells) for 24h to assess the phagocytic activity (%) **(F)** upon RV treatment of the monocytic fraction against target cells; **G)** Representative flow analysis and bar graph showing the co-localization between the GFP+ target cells and the CD14+ population and **H)** bar graph showing increase of the CD14+/GFP+ signal (%) in the total PBMC of the same 3 different HDs, upon CFZ treatment and RV infection (p=0.0006).

Combination of CFZ and RV promote cytotoxic T-Cell expansion in PBMC from Patients on Trial

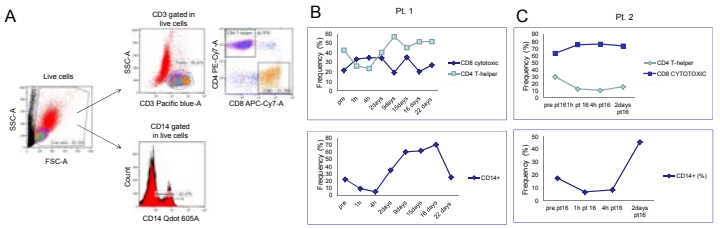

Fig.5 A-C) Pilot Clinical trial: RV + CFZ Pts were treated by IV infusion with CFZ 20mg/m^2 and after 30 minutes RV 3x10^{10} TCID$_{50}$ /day
A) Dot plot showing representative gating strategy for identification of CD3+ T cells (CD4+ and CD8+) and CD14+ Monocytes population gated in the live cells from MM patients in treatment with CFZ and RV from a pilot clinical trial; **B-C)** Line graphs showing the CD4+, CD8+ and CD14+ cell frequencies assessed at different time points, baseline-22 days (Pt.1 **B**) or baseline-2days (Pt.2 **C**), during combinatorial treatment with CFZ and RV.

Conclusion

- Carfilzomib enhances reovirus entry, infection, and killing of myeloma cells through its effect on the CD14+ fraction. Reovirus infection and replication within CD14+ cells is augmented because Carfilzomib inhibits the early innate pro-inflammatory immune response.
- Reovirus significantly increases CD14 frequency and activation/polarization in the monocytic fraction (CD14+), increasing the phagocytic activity against MM cells.
- Our data suggests that the combination Carfilzomib plus the oncolytic virus Pelareorep (Reovirus) increases the total frequency of cytotoxic T-cells.