Exploring the therapeutic potential of a peptide derived from a poxviral immune evasion protein

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Introduction

Vaccinia virus (VACV), a member of the poxvirus family and the active constituent of the vaccine that eradicated smallpox, has provided a vast collection of viral inhibitors of the host immune response. One poxviral protein A46 (Fig. 7 C) has been identified to inhibit TLR signalling by interacting with Toll-IL-1 receptor (TIR) domain containing proteins of the receptor complex to collectively inhibit all TLR adaptor proteins that positively regulate transcription-factor activation. Due to the ability of A46 to inhibit all TLRs [1] a small library of peptide fragments derived from this protein sequence was tested for ability to inhibit TLR signalling [2]. One 11 aa peptide (KYSFKLILAEY) termed VIPER was reported to retain the inhibitory properties of full length A46 against TLR4 signalling [2].

A 9R homopolymer delivery sequence at the C terminus provides delivery of the peptide into cells. The 9R delivery motif allows entry into cells where VIPER then targets TLR TIR domain adaptor proteins Mal and TRAM to inhibit TLR signalling. Mounting the 9R delivery sequence on the N terminus may offer better protection from proteosomal degredation and more efficient interaction with its adaptor protein targets. CP7 is an inert control peptide sequence (RNTISGNIYSA) also linked to a 9R homopolymer delivery sequence. This project investigates the affect of modifying the delivery sequence position. Peptide modification is used to elucidate some of the important properties required for activity. NMR is used for structural studies to identify important 3D confirmations of the peptide.

Abstract

Toll-like receptors (TLRs) have a role in viral detection leading to cytokine and IFN induction, and as such are targeted by viruses for immune evasion. The poxviral protein A46 has been identified to inhibit TLR signalling by interacting with TIR domain containing proteins of the receptor complex to collectively inhibit all TLR adaptor proteins that positively regulate transcription-factor activation. An inhibitory peptide derived from A46 termed VIPER selectively targets TLR4. This research investigates what affect the polyarginine delivery sequence location has on VIPER activity in vitro. In this study, 9R-VIPER is shown to be more effective at TLR4 and TLR2 signal inhibition than VIPER for multiple signalling pathways. Novel mechanisms of TLR2-driven interferon induction have been shown to be affected by 9R-VIPER and VIPER. The longevity of TLR4 inhibition by 9R-VIPER was shown to be superior to VIPER. Residues required for peptide activity were identified. Structural information about 9R-VIPER and a loss-of-function mutant was determined by NMR analysis.

Project Aims:

- Compare the ability of VIPER and 9R-VIPER to block TLR4 and TLR2 agonist responses in vitro.
- Determine the longevity of TLR4 inhibition by VIPER and VIPER-derived peptides in vitro.
- Compare wild type and peptides with muted residues predicted to be required for protein binding.
- Determine the NMR structure of 9R-VIPER.

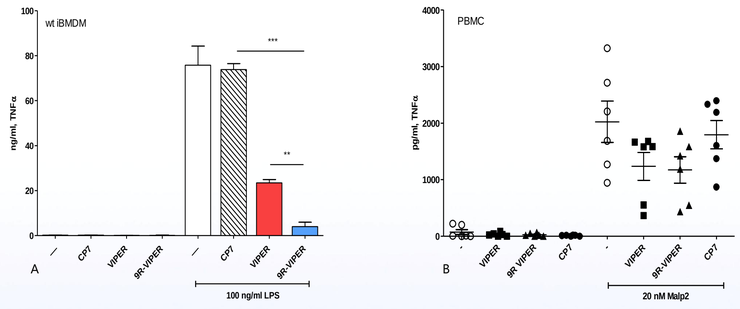
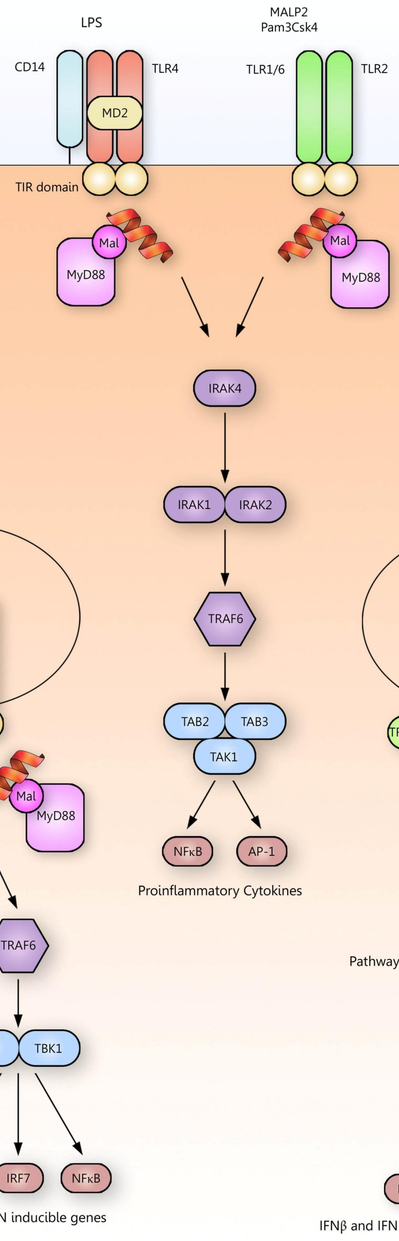


Fig.1 9R-VIPER has greater inhibitory properties than VIPER for LPS driven TNFα in both wt iBMDM and human PBMC. TLR4 signals from the plasma membrane and endosomal compartment for induction of pro-inflammatory cytokines. [3] Wt iBMDM and multiple human PBMC donor samples were treated with peptides VIPER, 9R-VIPER and CP7 at multiple concentrations 1 hour before stimulation with 100 ng/ml LPS. Supernatants assayed for TNFα by ELISA. Representative of 5 and 20 μM conc of peptides in BMDM and PBMC respectively. Mean of triplicate samples, representative of >3 independent experiments.



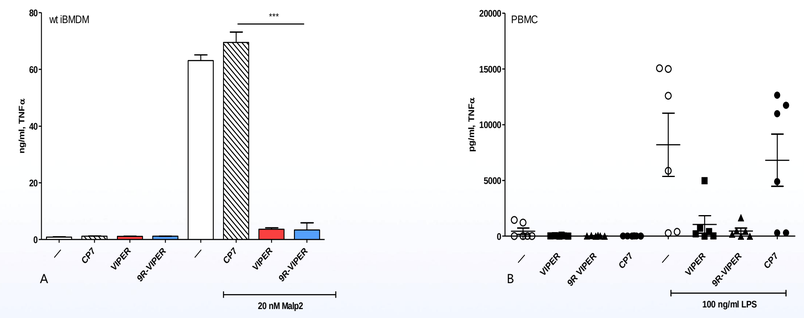


Fig. 3 9R-VIPER has greater inhibitory properties than VIPER for Malp2 driven TNFα in wt iBMDM and human PBMC. TLR2 signalling induces dose dependent Mal interaction with MyD88 for production of pro-inflammatory cytokines from the plasma membrane although MyD88 involvement is dose dependent [4]. Wt iBMDM and human PBMC from multiple donors were treated with peptides VIPER, 9R-VIPER and CP7 at multiple concentrations prior to stimulation with 20 nM Malp2. Supernatants were harvested and assayed for TNFα. Shown here are 5 and 20 μM conc of peptides in BMDM and PBMCM respectively, in triplicate samples and representative of experiments replicated independently.

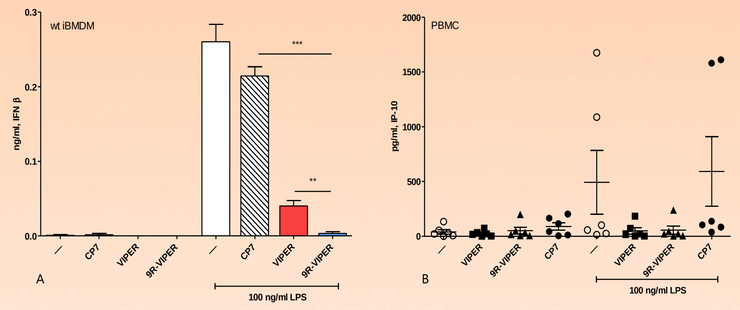


Fig.2 9R-VIPER has greater inhibitory properties than VIPER for LPS driven IFNβ in wt iBMDM and IP-10 in human PBMC. IFN related genes are induced via TLR4 signalling after receptor endocytosis. Wt iBMDM and human PBMC from multiple donors were treated with peptides VIPER, 9R-VIPER and CP7 at multiple concentrations 1 hour before stimulation with 100 ng/ml LPS. Supernatants were harvested and assayed for IFNβ and IP-10 by ELISA. These results represent 5 and 20 μM conc of peptides in BMDM and PBMCM respectively. Mean of triplicate samples, representative of repeatable data.

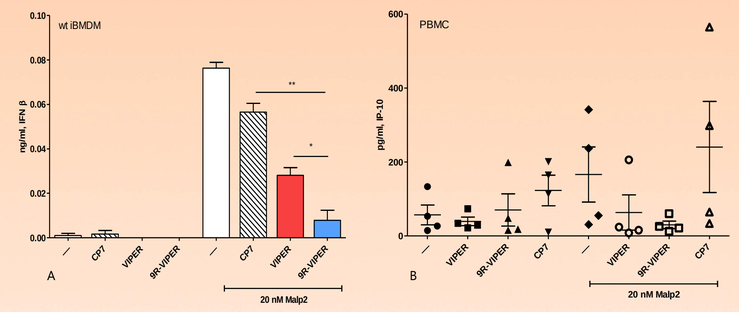


Fig. 4 9R-VIPER has greater inhibitory properties than VIPER for Malp2 driven IFNβ in wt iBMDM and human PBMC. Previously, IFN related genes were not thought to be induced by TLR2 ligand binding [5]. Here we have shown that not only is IFNβ induced via TLR2 but 9R-VIPER is a potent inhibitor of this signalling pathway. The question of adaptor protein involvement for TLR2-driven interferon induction has been a mystery for several years. Mal dependency is thought to be determined by the level of ligand stimulation, yet 9R-VIPER is shown to inhibit adaptor binding more potently than VIPER. Wt iBMDM and human PBMC treated with peptides VIPER, 9R-VIPER and CP7 at multiple concentrations prior to stimulation with 20 nM Malp2. Supernatants assayed for IFNβ and IP-10 respectively. Shown here are 5 and 20 μM conc of peptides in BMDM and PBMCM respectively, in triplicate samples and representative of experiments replicated independently.

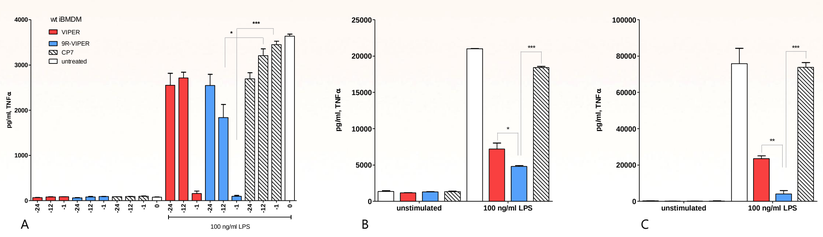
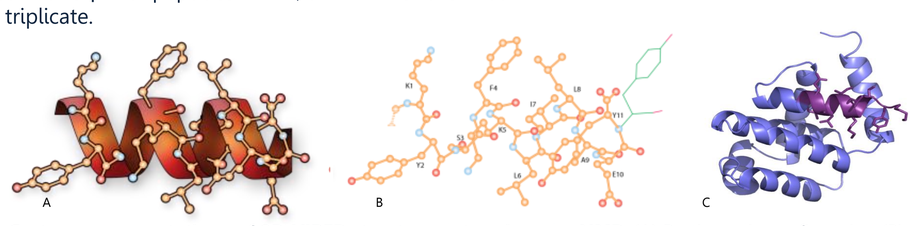


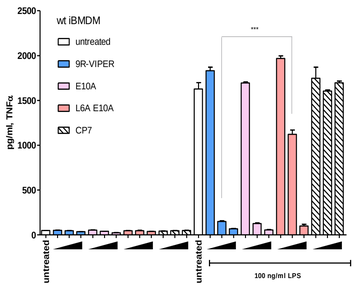
Fig. 5 9R-VIPER has greater longevity for TLR4 inhibition than VIPER in vitro. The application of VIPER as an anti-inflammatory therapeutic requires a knowledge of the longevity of biological activity. (A) When delivered shortly before lignad stimulation 9R-VIPER has greater inhibition than VIPER a desirable trait for preventing ischemia/reperfusion injury. A method called retro-inverso modification may be applied to 9R-VIPER, which prolongs the half-life of peptides due to their resistance to proteosomal degradation. Once stimulation occurs, 9R-VIPER shows greater inhibitory properties than VIPER for extended periods of (B) 3 hr and (C) 24 hr stimulation. wt iBMDM (seeded at $1x10^5$ cells/ml) were treated with 5 μM of peptides VIPER, 9R-VIPER and CP7 at multiple intervals prior to 100 ng/ml LPS stimulation. Representative figures of multiple experiments in triplicate.

Fig. 6 9R-VIPER peptide residues leucine 6 and glutamic acid 10 are required for effective TLR4 signal inhibition. Because of the significant improvements that the N- rather than C-terminal 9R had on VIPER activity, 9R-VIPER was selected for further mutagenesis studies. Residues believed to be important for adaptor protein binding were investigated. Mutant peptides were synthesised with alanine substitutions (with their simple short sidechain methyl group) in place of leucine 6 and/or glutaminc acid 10. The peptide with double mutated residues L6 and E10 looses significant inhibitory activity. wt iBMDM and human PBMC were treated with 5 μM of peptides 9R-VIPER, both mutant peptides E10A and L6AE10A, and CP7 1 hr prior to 100 ng/ml LPS stimulation. Representative figure from wt iBMDM. Concurrent results were seen in human PBMC (not shown).

Fig. 7 NMR structural studies. Because of the success of 9R-VIPER inhibitory properties, NMR studies were used to investigate the 3D structure of both 9R-VIPER and the mutant L6AE10A. Detailed insight into the backbone and side-chain configurations were found to explain the partial α-helix confirmation of 9R-VIPER. The L6E10A mutant peptide was found to have a simpler helical confirmation. This is due to the loss of the long side chains of tyrosine and leucine whos α, β, γ, and δ, protons in particular were used stabilise the more complex confirmation of 9R-VIPER. These differences after residue substitution explain the loss of inhibitory activities in vitro for the mutant peptide.



Cartoon representations of 9R-VIPER structure determined by NMR. (A) Partial helix confirmation (B) Residues are labelled. Green wireframe shows tyrosine 10 confirmation when VIPER is present on A46 [7]. (C) Structure of the protein A46 with the VIPER motif shown in purple [6].



In conclusion, the work presented here confirms:

- 9R-VIPER is an effective at TLR4 and TLR2 signal inhibition than VIPER.
- Novel mechanisms of TLR2-driven interferon induction are affected by 9R-VIPER and VIPER.
- 9R-VIPER has greater longevity for TLR4 inhibition than VIPER in vitro.
- Amino acid residues important for 9R-VIPER inhibitory activity were identified.
- The structure of 9R-VIPER and the mutant peptide L6AE10A were investigated by NMR.
- Important structural information was uncovered and an explanation was found for the loss of activity when important residues are changed.

Significance

The results presented here provide insights into the structure and activity of 9R-VIPER. This information will be used to select optimal peptides for further in vivo efficacy studies. The NMR structure of these peptides will be applied in moving to design peptidomimetics based on VIPER. Overall the project has contributed to the development of a novel virally-derived TLR4 inhibitor which may ultimately have use in TLR4-dependent human disease.

References

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