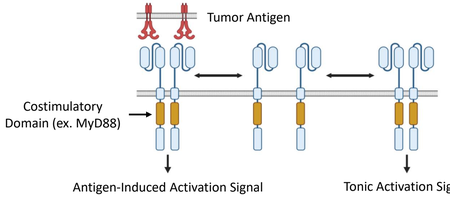
Redirected Soluble Modulators (RSM) – A Novel Engineering Strategy to Enhance Immune Receptor Signaling

Nicholas G. Minutolo, Lauren C. Shaw, Tierra S. Tobin, Kerri Ciccaglione, Robert J. Saporito, Benjamin H. Schott, Rehman Qureshi, Thomas Condamine, Michael Klichinsky

Carisma Therapeutics Inc., Philadelphia, PA

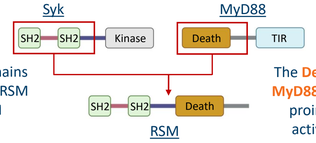
Abstract

Immune cells require activation of primary and secondary signaling pathways to achieve maximum effect. To harness secondary signals in the design of synthetic immune receptors such as chimeric antigen receptors (CARs), costimulatory domains have been grafted directly into the membrane proximal intracellular space of these receptors. While direct incorporation of co-stimulatory domains such as 4-1BB and CD28 has been a successful strategy in CAR-T cell design, there are certain signaling domains for which membrane localization can lead to constitutive (tonic) signal propagation. This challenge is particularly notable for macrophages which signal through innate immune pathways such as MyD88 which activate from membrane proximity.



To overcome the hurdle of tonic signaling from costimulatory domains, we developed redirected soluble modulators (RSMs). These chimeric soluble proteins combine the phospho-tyrosine binding capabilities of Src Homology 2 (SH2) domains from one protein with signal-transducing portions of another. Here we demonstrate an RSM that combines the phospho-ITAM binding SH2 domains from Syk with the signaling death domain of MyD88. The Syk-MyD88 RSM is capable of localizing to an ITAM domain that endogenously recruits Syk, but upon recruitment drives MyD88 signaling alongside endogenous Syk signaling. We co-expressed an ITAM-containing CAR together with the Syk-MyD88 RSM in macrophages. Upon antigen-induced CAR activation, the Syk-MyD88 RSM greatly enhanced proinflammatory cytokine output while simultaneously driving an expansion of the proinflammatory cytokine profile and enhancing M1 polarization compared to CAR activation alone. When the Syk SH2 domains of the RSM were mutated, the effect was lost, confirming the necessity for recruitment of the RSM to the ITAM to drive function. Additionally, the Syk-MyD88 RSM abrogated the tonic, antigen-independent release of proinflammatory cytokines seen with CARs that contain MyD88 death domain directly in the CAR construct. Syk-MyD88 RSMs enhanced the anti-tumor activity of CAR macrophages (CAR-M).

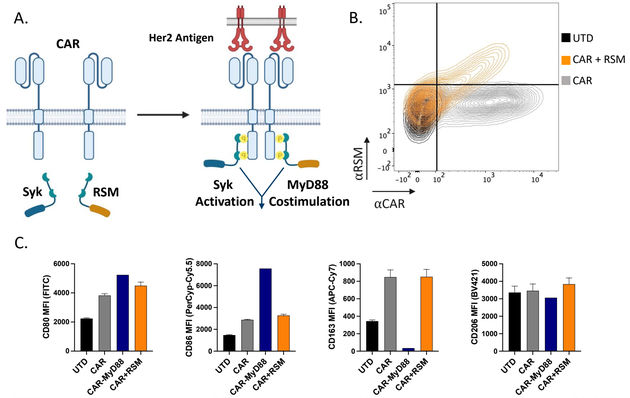
Macrophages are critical effector cells of monoclonal antibodies. We evaluated the ability of Syk-MyD88 RSM to redirect endogenous Fc receptors (FcRs) to activate MyD88 in response to antibody signaling as a strategy to endow antibodies with multi-pronged innate immune signaling. When activated with antigen-specific monoclonal antibodies such as trastuzumab, macrophages expressing the Syk-MyD88 RSM exhibited a marked increase in antigen specific cytokine release, an expanded proinflammatory cytokine/chemokine output profile, and enhanced M1 polarization. RSM macrophages stimulated with antibodies activated MyD88 signaling, and thus offer a universal strategy to enhance monoclonal antibody-based therapy.



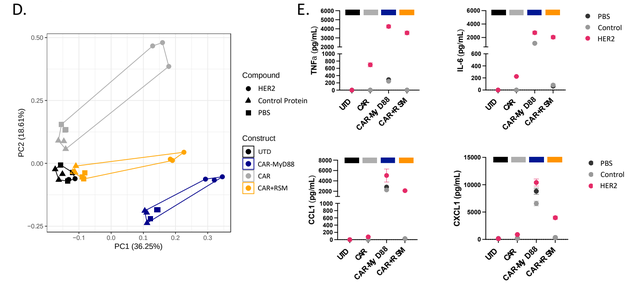
SH2 recruitment domains from **Syk** localize the RSM to activated ITAM domains

The **Death Domain** of **MyD88** elicits a strong proinflammatory activation signal

RSMs alleviate tonic MyD88 signaling and provide antigen-dependent co-stimulation upon CAR activation

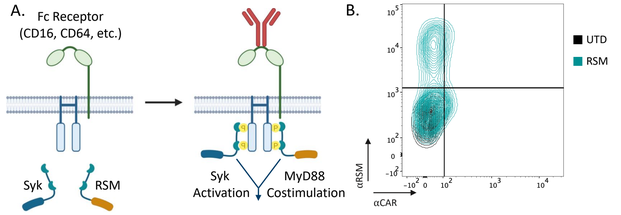


RSMs can be co-expressed with CAR and alleviate tonic M1 polarization caused by surface MyD88 localization - A. Mechanism of action for RSMs in the context of CAR activation. B. Expression of first-generation anti-HER2 CAR (grey) vs. CAR+RSA (orange). C. Flow analysis of M1 (CD80, CD86) and M2 (CD163, CD206) markers on the surface of macrophages. CARs containing MyD88 costimulation exhibit tonic M1 polarization, while CARs co-expressed with RSM show no change in polarization compared to CAR-only control.

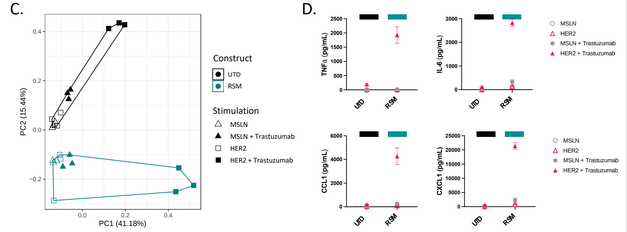


RSM recruitment to activated CAR potentiates proinflammatory cytokine secretion and enhances cytokine profile – D. Principal Component Analysis (PCA) plot based on broad panel cytokine secretion analysis of macrophages stimulated with either PBS, control antigen (mesothelin), or target antigen (HER2). E. Individual plots of select proinflammatory cytokines and chemokines from broad panel analysis plotted for each macrophage. CARs containing MyD88 costimulation secrete proinflammatory cytokines in an antigen-independent manner. RSMs expand the cytokine profile of CAR activation, while mitigating non-specific cytokine secretion.

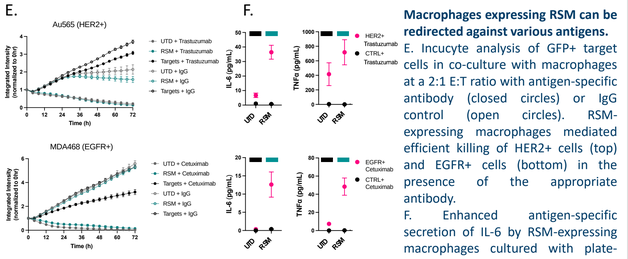
RSMs act as a co-stimulatory molecule for endogenous Fc Receptors



RSMs can function independently of CAR to signal via FC receptor activation. A. Mechanism of RSM recruitment to phosphorylated FC receptor ITAMs. B. Expression of RSM (teal) compared to untransduced (UTD, black) macrophages measured by flow cytometry.

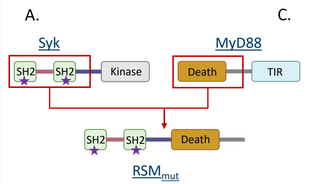


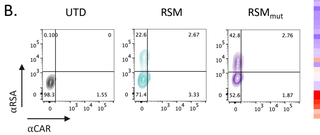
Macrophages expressing RSM have unique cytokine profile upon stimulation via ADCC. C. PCA plot based on broad panel cytokine analysis after 24-hour culture with plate-bound antigen. D. Individual plots of cytokines demonstrating enhanced secretion upon stimulation with HER2 in the presence of Trastuzumab (pink, closed triangle). Mesothelin (MSLN, gray) was used as a non-targeted control.

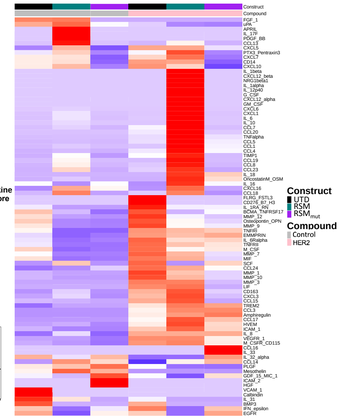


Macrophages expressing RSM can be redirected against various antigens.
E. Incucyte analysis of GFP+ target cells in co-culture with macrophages at a 2:1 E:T ratio with antigen-specific antibody (closed circles) or IgG control (open circles). RSM-expressing macrophages mediated efficient killing of HER2+ cells (top) and EGFR+ cells (bottom) in the presence of the appropriate antibody.
F. Enhanced antigen-specific secretion of IL-6 by RSM-expressing macrophages cultured with plate-bound antigen over 24 hours.

Mutations to prevent RSM ITAM localization abrogate activation



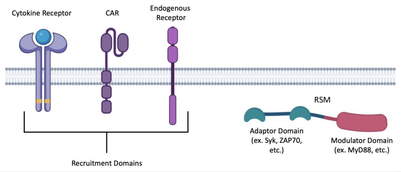




Mutations in the RSM SH2 domains prevent ITAM localization and eliminate costimulation effect. A. Schematic of RSM_{mut}. B. Expression of RSM (teal) vs. RSM_{mut} (purple). C. Heat map of cytokine secretion demonstrating loss of MyD88 specific activation in RSM_{mut} compared to RSM. All conditions were in the presence of Trastuzumab.

Conclusions

- RSMs allow for the decoupling of costimulatory domains from the CAR construct, helping alleviate issues with constitutive signaling, while maintaining the antigen-dependent activation of costimulatory signals
- Syk-MyD88 RSMs provide a universal method to rewire monoclonal antibody Fc receptor signaling to boost macrophage activation and anti-tumor function
- RSMs have cell type agnostic potential to rewire signaling downstream of TCRs (T cell receptors), FcRs, CARs, and other endogenous immunoreceptors



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