Surrogate Cytokines Agonists (SCAs): a novel combinatorial array of biased surrogate cytokine agonists with antibody-like druggability

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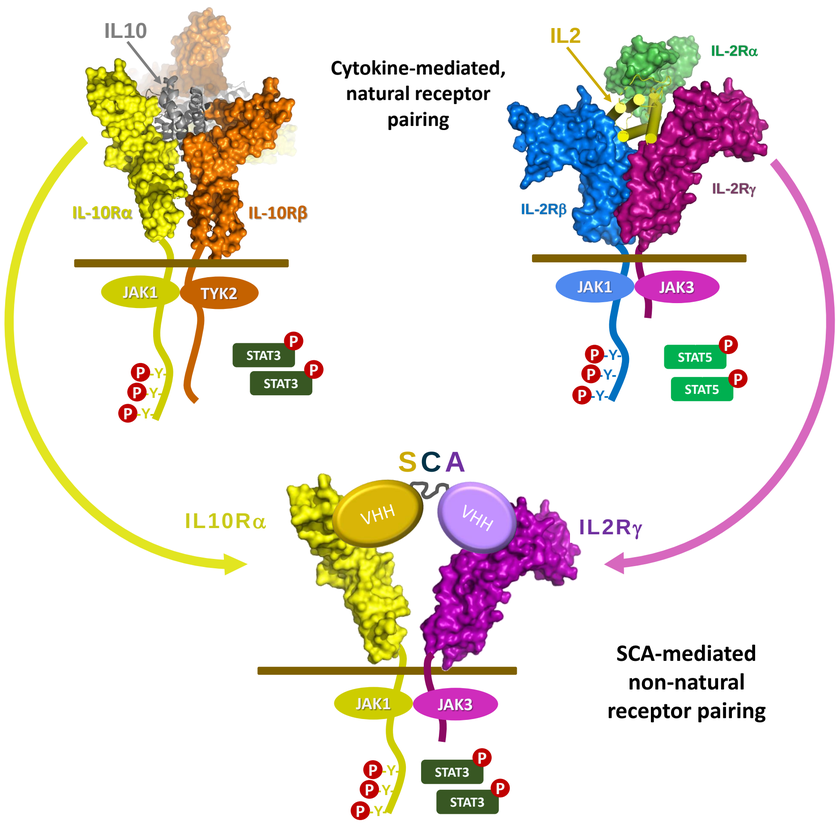
Abstract: Creating Natural and Non-Natural Cytokine Signals via Dual VHH SCAs

Cytokines are secreted proteins that activate signaling pathways in immune cells by bringing together two or more receptors. Their use as therapeutics is limited by the cognate receptors they can engage and by the pleiotropy that these receptors exhibit across multiple cell types. We have sought to overcome these limitations using single-domain (VHH) antibodies to multimerize both native and non-native pairs of cytokine receptors. This allows us to modulate naturally occurring signals as well as create new ones aimed at specific cell types. Here we describe three examples, in which we recapitulate the activity of Interleukin-10 (IL-10) and Interleukin-2 (IL-2) as well as create a non-natural signal derived by the hybridization of the two receptors. VHHs specific for human IL-10Rα, IL-10Rβ, IL-2Rβ and IL-2Rγ were generated by llama immunization and screening of VHH libraries prepared from peripheral blood. Seven IL-10Rα VHHs and seven IL-10Rβ VHHs were combined as expression fusions in an all-by-all matrix and in both amino/carboxy terminal orders, thus yielding 98 unique surrogate cytokines agonists (SCAs). Similarly, twelve IL-2Rβ and six IL-2Rγ VHHs were combined into 144 SCAs. Eleven IL-10Rα and six IL-2Rγ VHHs yielded 84 unique SCAs. After screening on reporter cell lines, all three combinations returned a selection of SCAs able to generate activity in primary cells. IL-10 SCAs induced pSTAT3 phosphorylation in human monocytes, B cells, NK cells, CD4+/CD8+ T cells with varying signaling strengths. They also inhibited LPS-induced secretion of IL1β and TNFα by monocytes but were less potent at inducing IFN-γ and granzyme production in T cells, thus demonstrating the ability to decouple the immunosuppressive and immunostimulatory activities of IL10. IL-2 SCAs were able to stimulate varying levels of pSTAT5 phosphorylation, proliferation and IFNγ secretion in both NK and CD4+/CD8+ T cells. Finally, the IL-10/IL-2 hybrid SCAs induced pSTAT3 signal in B cells, NK cells, CD4+/CD8+ T cells with little to no pSTAT3 signal in monocytes. They also induced proliferation and Granzyme production by CD8+ T cell blasts generated upon CD3/CD28 activation. Consistent with the lack of STAT3 signaling in monocytes, these IL-10/IL-2 SCAs did not inhibit LPS-induced secretion of IL1β and TNFα by monocytes, suggesting selectivity and a lack of immunosuppressive activities. We believe this platform will enable a rapid, combinatorial expansion of both existing and novel cytokine signaling solutions for specific immune cells of interest.

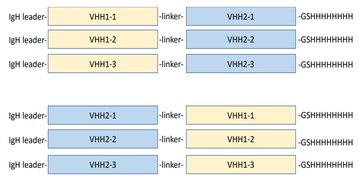
Figure 1. Cytokine-mediated and SCA-mediated receptor pairing and downstream signaling



Generation of IL-2Rβ/IL-2Rγ, IL-10Rα/IL-10Rβ, IL-10Rα/IL-2Rγ SCAs

The extracellular domains of human IL-2Rβ, IL-2Rγ, IL-10Rα and IL-10Rβ were expressed with human IgG1 Fc and used to immunize camels. Heavy chain single domain antibody (VHH) libraries prepared from PBMCs were screened for binding the immunogens. Individual, unique VHH sequences specific for each receptor were paired together in both orientations separated by a short linker to obtain panels of dual VHHs, here referred to as SCAs. A C-terminal His_8 tag was added to facilitate purification.

Figure 2.
Assembly of SCAs in two orientations.



Binding kinetics of VHH domains

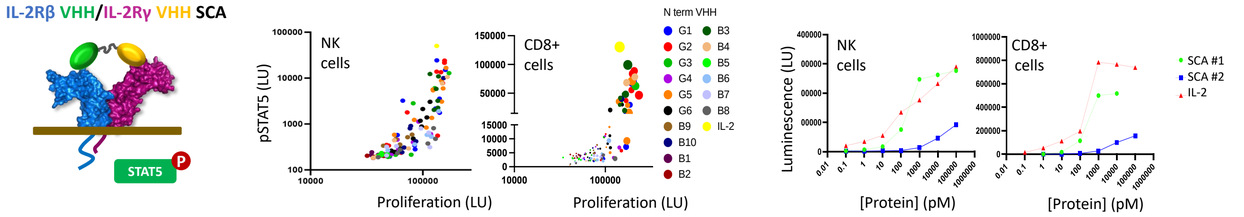
Table 1 (below) provides kinetics data summarizing association and dissociation rate constants and affinity constants for each individual VHH. The experiments were conducted on a Biacore™ T200 instrument equipped with a Protein A chip. VHH-Fc fusions were captured on the chip and IL-2Rβ or IL-2Rγ or IL-10Rα or IL-10Rβ extracellular domains were injected at concentrations ranging from 1.2 to 400 nM. Measurements were performed in single cycle kinetics mode.

Table 1. Binding kinetics of IL-2Rβ, IL-2Rγ, IL-10Rα and IL-10Rβ specific VHHs

Receptor	k_{ON} (1/Ms)	k_{OFF} (1/s)	Affinity (nM)
IL-10Rα	1.0E+05	1.4E-03	13
	1.1E+05	1.9E-04	1.8
	1.7E+05	1.1E-03	6.4
	9.9E+04	1.2E-03	12
	1.5E+05	1.2E-03	7.7
	9.8E+04	1.4E-04	1.4
	3.3E+05	2.0E-03	6
IL-10Rβ	1.8E+05	1.4E-03	8.3
	1.1E+05	1.4E-03	12.9
	9.3E+04	2.1E-02	231
	7.2E+04	8.7E-03	121
	1.1E+05	5.8E-04	5.5
	6.8E+04	2.1E-02	314
	1.1E+07	2.5E-02	2.4
IL-2Rβ	2.0E+07	2.0E-02	1
	1.6E+05	7.0E-03	47
	6.0E+05	2.1E-03	3.4
	1.4E+05	2.2E-03	16
	3.8E+06	1.5E-03	0.4
	1.9E+07	2.3E-02	1.2
	2.9E+06	3.2E-03	1.1
IL-2Rγ	2.0E+07	2.0E-02	1
	1.6E+05	7.0E-03	47
	6.0E+05	2.1E-03	3.4
	1.4E+05	2.2E-03	16
	3.8E+06	1.5E-03	0.4
	1.9E+07	2.3E-02	1.2
	2.9E+06	3.2E-03	1.1

IL-2Rβ/IL-2Rγ SCAs activity in NK cells and CD8+ T cells derived from primary peripheral blood

Figure 3. IL-2Rβ/IL-2Rγ SCAs activity in human NK cells and CD8+ T cells

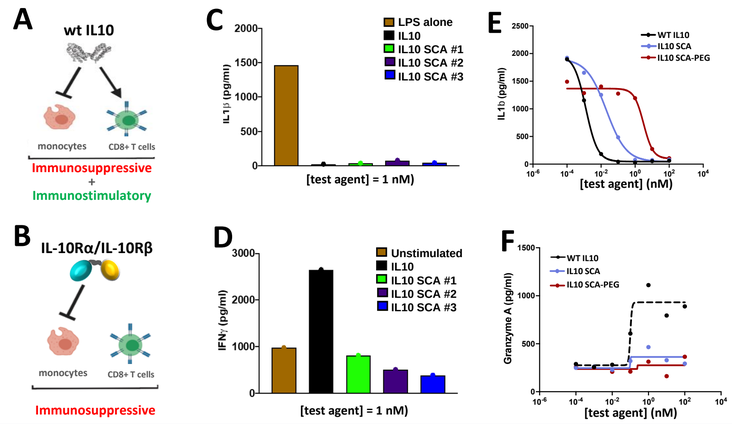


(A) A schematic representation of an IL-2Rβ/IL-2Rγ SCA dimerizing IL-2Rβ and IL-2Rγ resulting in the IL-2-like activation of cells expressing the intermediate affinity IL2 receptor. (B) STAT5 phosphorylation and proliferation of NK cells isolated from peripheral blood and treated with IL-2Rβ/IL-2Rγ SCAs. In the multivariate plot, each dot represents an individual VHH dimer with different colors indicating VHH dual dimers grouped by N terminal VHH segment used. (C) Interferon gamma (IFN-γ) secretion in NK cells (left) and CD8+ T cells (right) isolated from peripheral blood and treated with two IL-2Rβ/IL-2Rγ SCAs or IL-2.

IL-10Rα/IL-10Rβ SCAs inhibit cytokine secretion without activating T cells

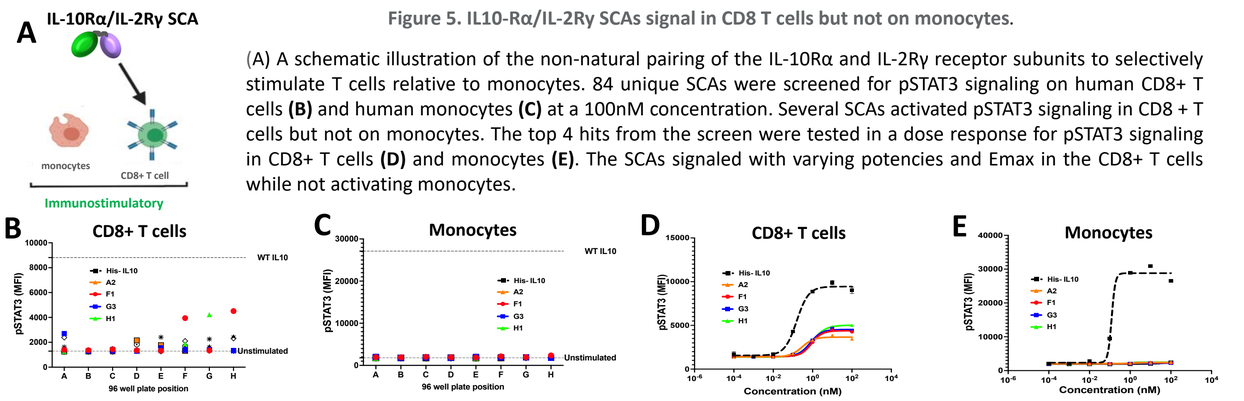
(A) IL-10 exemplifies cytokine pleiotropy by suppressing inflammatory monocytes while simultaneously activating CD8+ T cells. **(B)** Using an IL-10Rα/IL-10Rβ SCA, we uncouple the immunosuppressive and immunostimulatory properties of IL10 via modulation of signaling strength (*i.e.*, partial agonism). **(C)** Following LPS shock of monocytes, three different IL-10Rα/IL-10Rβ SCAs exhibit similar inhibition of IL-1β secretion compared to IL10. **(D)** In contrast to IL-10, three IL-10Rα/IL-10Rβ SCAs do not induce IFN-γ secretion in CD8+ T cells. **(E)** Dose response inhibition of IL-1β secretion in LPS-shocked monocytes with an IL-10Rα/IL-10Rβ SCA, a PEGylated version of the IL-10Rα/IL-10Rβ SCA and human IL-10. **(F)** Dose response expression of granzyme A for the three molecules described in the top panel. Similar results are obtained for TNFα and IL-6 secretion in monocytes and for granzyme B in CD8+ T cells (not shown).

Figure 4. Partial agonism of IL-10Rα/IL-10Rβ SCA on monocytes and CD8+ T cells



IL-10Rα/IL-2Rγ SCAs selectively activate T cells over monocytes

Figure 5. IL10-Rα/IL-2Rγ SCAs signal in CD8 T cells but not on monocytes.



(A) A schematic illustration of the non-natural pairing of the IL-10Rα and IL-2Rγ receptor subunits to selectively stimulate T cells relative to monocytes. 84 unique SCAs were screened for pSTAT3 signaling on human CD8+ T cells **(B)** and human monocytes **(C)** at a 100nM concentration. Several SCAs activated pSTAT3 signaling in CD8 + T cells but not on monocytes. The top 4 hits from the screen were tested in a dose response for pSTAT3 signaling in CD8+ T cells **(D)** and monocytes **(E)**. The SCAs signaled with varying potencies and Emax in the CD8+ T cells while not activating monocytes.

Conclusions

We have generated a series of functional synthetic cytokine agonists (SCAs) that mimic and modulate natural signals or create new ones.

- IL-2 mimicking SCAs can recapitulate IL-2 functions: induce STAT5 phosphorylation, IFNγ production and proliferation of NK and CD8+ cells.
- IL-10 partial agonist SCAs exhibit a signaling bias on monocytes over T cells.
- IL-10Rα/IL-2Rγ SCAs activate T cells, while remaining silent on monocytes.

The multimerization of natural and unnatural combinations of cytokine receptors via SCAs the potential to generate a vast repertoire of natural or novel immunomodulatory signals.