#4225. IL-2Rβ/IL-2Rγ Synthetic Cytokines Induce Activation of Human T and NK Cells.

Rene de Waal Malefyt, PJ Aspuria, Sandro Vivona, Priyanka Balasubrahmanyam, Cindy Buffone, Mahalakshmi Ramadass, Romina Riener, Martin Oft, Patrick Lupardus and Robert Kastelein.

Synthekine

1505 O'Brien Dr, Menlo Park, CA, 94025

Introduction: Heterodimerization of IL-2Rβ/IL-2Rγ initiates IL-2 signaling pathways

Heterodimerization of the intermediate affinity Interleukin-2 Receptors (IL-2R), IL-2Rβ and IL-2Rγ, initiates a signaling cascade in T and NK cells that ultimately results in proliferation and production of Interferon-gamma (IFN-γ). Binding of IL-2 in a dimeric complex with intermediate affinity or in the high affinity trimeric complex, including IL-2Rα, on activated T cells and Tregs leads to trans-phosphorylation of signaling motifs on the IL-2Rβ and IL-2Rγ intracellular domains by the associated JAK kinases. The function of the ligand is to bring the receptor chains in close proximity to produce an optimal level of phosphorylation and activation of associated STAT transcription factors. Here we established a way to bring IL-2Rβ and IL-2Rγ chains together in a cytokine-independent manner that results in functional activation.

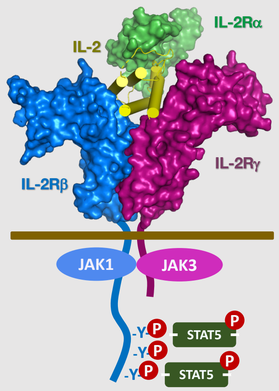

Figure 1. IL-2-IL-2R signaling complex

Generation and construction of IL-2Rβ and IL-2Rγ dual VHH dimers

Extracellular domains of human IL-2Rβ and IL-2Rγ were expressed with human IgG1 Fc and used to immunize camels. Heavy chain single domain antibody (VHH) libraries prepared from PBMC were screened for binding the immunogens. Ten unique VHH sequences from IL-2Rβ selections and six unique VHH sequences from IL-2Rγ selections were paired together in both orientations separated by a short linker to obtain a total of 120 dual VHH dimers. A C-terminal his tag was added to facilitate purification.

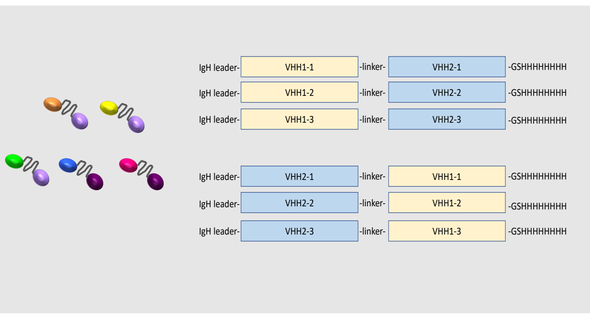

Figure 2. IL-2Rβ/IL-2Rγ dual VHH dimers.

Binding kinetics of IL-2Rβ and IL-2Rγ specific VHH segments

Ligand	k_{on} (1/Ms)	k_{off} (1/s)	Affinity K_D (nM)
IL-2Rβ	3.8E+06	1.5E-03	**0.4**
	2.0E+07	2.0E-02	**1**
	2.9E+06	3.2E-03	**1.1**
	1.9E+07	2.3E-02	**1.2**
	6.0E+05	2.1E-03	**3.4**
	1.0E+04	1.0E-04	**10**
	1.4E+05	2.2E-03	**16**
	1.6E+05	7.0E-03	**47**
	1.7E+04	1.9E-03	**503**
IL-2Rγ	3.7E+05	8.1E-04	**2.2**
	2.9E+06	6.9E-03	**2.4**
	5.5E+05	3.5E-03	**6.4**
	1.9E+05	2.7E-03	**14**
	6.6E+04	1.1E-03	**17**
	9.7E+04	2.5E-03	**26**

Table 1. Binding kinetics of IL-2Rβ and IL-2Rγ specific VHH's

Kinetics table summarizing association and dissociation rate constants and affinity constants for each VHH. IL-2Rβ and IL-2Rγ VHH affinities are in the low nanomolar range. 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 extracellular domains were injected ranging from 1.2 to 400 nM. Measurements were performed in single cycle kinetics mode.

IL-2Rβ/IL-2Rγ dual VHH dimers activate NKL cells

NKL cells are an IL-2 dependent human cell line that expresses IL-2Rβ and IL-2Rγ chains and can respond to IL-2 by phosphorylation of STAT5 and proliferation. IL-2Rβ/IL-2Rγ dual VHH dimers displayed varied IL-2 agonist activities and induced STAT5 phosphorylation (MSD) (Y-axis) at 20 min and proliferation (CTG) (X-axis) at 72 hrs. 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.

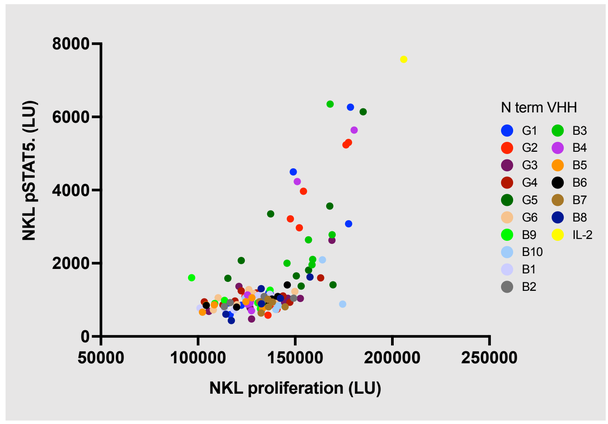

Figure 3. IL-2Rβ/IL-2Rγ dual VHH dimers induce pSTAT5 and NKL proliferation. (LU = Luminescence Units)

IL-2Rβ/IL-2Rγ dual VHH dimers activate primary peripheral blood NK cells

NK cells were isolated from peripheral blood by positive selection using CD56 microbeads (Miltenyi) and tested for responsiveness to IL-2Rβ/IL-2Rγ dual VHH dimers displayed varied IL-2 agonist activities and induced pSTAT5 phosphorylation (MSD) (Y-axis) at 20 min and proliferation (CTG) (X-axis) at 72 hrs. 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. IL-2 was used as positive control.

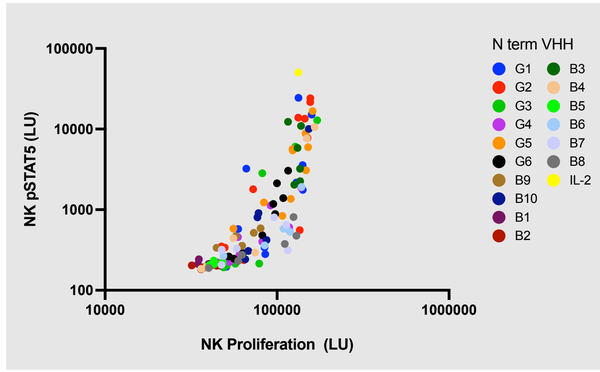

Figure 4. IL-2Rβ/IL-2Rγ dual VHH dimers induce pSTAT5 and proliferation of human NK cells. (LU = Luminescence Units)

Potency of IL-2Rβ/IL-2Rγ dual VHH dimers on primary peripheral blood NK cells

IC50 values and emax values were determined for STAT5 phosphorylation and IFNγ production on purified human NK cells for 42 of the IL-2Rβ/IL-2Rγ VHH dimers that showed the greatest activity in single concentration assay screens. IL-2Rβ/IL-2Rγ VHH dimers were
titrated down from 100 nM in 10 fold dilutions to determine IC50's and emax relative to the response to IL-2.
IL-2Rβ/IL-2Rγ dimers displayed a varied range of potencies in both IC50 (X-axis) and emax (Y-axis) with a number of VHH's having lower IC50's compared to IL-2 for STAT5 phosphorylation, but not for IFN-γ production.

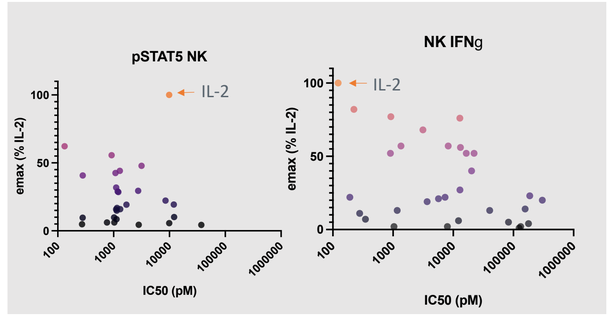

Figure 5. IL-2Rβ/IL-2Rγ dual VHH dimers induce pSTAT5 and IFNγ production of human NK cells.

IL-2Rβ/IL-2Rγ dual VHH dimers activate primary activated peripheral blood CD8 positive T cells

Peripheral Blood Mononuclear Cells were isolated and cultured with anti-CD3 and anti-CD28 mAbs for 72 hrs. Cells were harvested and CD8 positive T cells were isolated by positive selection using CD8 microbeads (Miltenyi). Activated CD8 positive T cell blasts were incubated with IL-2Rβ/IL-2Rγ dual VHH dimers for 20 min and STAT5 phosphorylation was measured in cell lysates using the MSD phosphoSTAT panel kit. Results are represented on the Y-axis. In addition, CD8 positive T cells were incubated with IL-2Rβ/IL-2Rγ dual VHH dimers for 72 hrs, cell culture supernatants were harvested, and proliferation measured using the Cell Titer Glow kit (Promega) (X-axis). IFN-γ levels in the supernatants were determined using MSD human IFN-γ kit. The size of each dot is proportional to the levels of IFN-γ production. IL-2 was used as positive control.

Forty two of the 120 IL-2Rβ/IL-2Rγ dual VHH dimers induced significant STAT5 phosphorylation, proliferation and IFN-γ production of activated CD8 positive T cell blasts. 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. IL-2Rγ VHH segments G2 and G5 show activity when paired with multiple IL-2Rβ segments. There is a good correlation between pSTAT5 induction, proliferation and IFN-γ production by IL-2Rβ/IL-2Rγ dual VHH dimers. (LU = luminescence units)

Similar results were obtained when CD4 positive T cells were used.

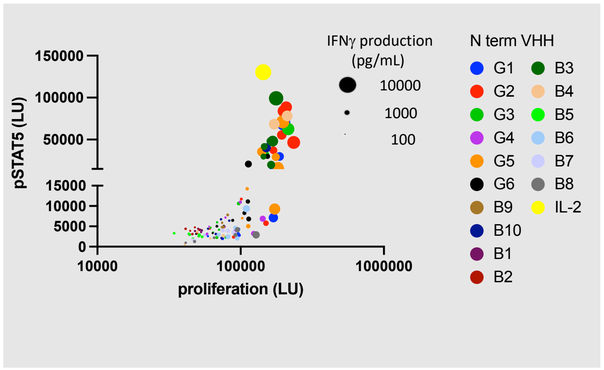

Figure 6. IL-2Rβ/IL-2Rγ dual VHH dimers induce pSTAT5, proliferation and IFNγ production of human CD8⁺ T cells.

Conclusions

We have generated a series of functional IL-2Rβ/IL-2Rγ synthetic cytokines each with unique signaling capacity that are

- Active on NKL cells and induce STAT5 phosphorylation and proliferation.
- Active on primary NK cells and induce STAT5 phosphorylation, proliferation and IFN-γ production.
- Active on primary activated CD4 and CD8 positive T cell blasts cells and induce STAT5 phosphorylation, proliferation and IFN-γ production