



Following T-cell activation and differentiation
with HTRF reagents: IL-2, IFN- γ and IL-17

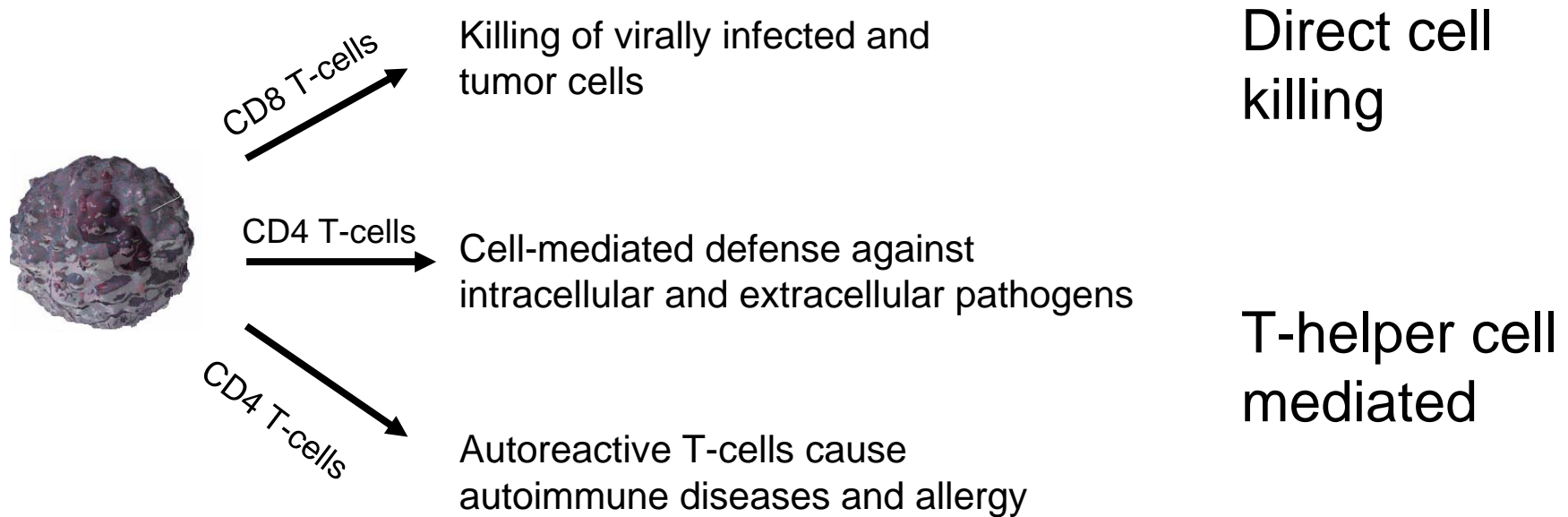
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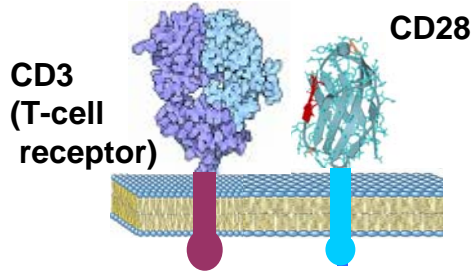
Introduction: T-cells have effector and helper functions in the immune system

T-cells participate in immune reactions either as effector cells (direct cell killing) or helper cells (attracting and activating lymphocytes)

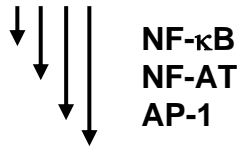
Their role is normally beneficial but can become harmful when auto-reactive T-cells escape immune control and cause immune mediated tissue damage (manifestations are very often specific for one organ!)



Interleukin-2 (IL-2) is a reporter for T-cell activation



Signaling cascades downstream TCR lead to gene induction

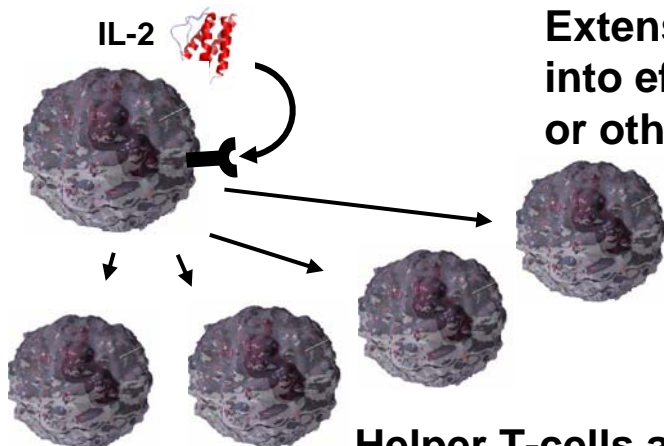


The human T-cell repertoire contains 10^{12} cells, which are composed of 10^{8-9} different T-cell clones (only 10^3 to 10^4 T-cells per clone).

Therefore, the first step after T-cell activation is clonal expansion.

Engagement of the T-cell Receptor CD3 (Signal 1) and the co-stimulatory molecule CD28 (Signal 2) is sufficient to induce limited clonal expansion of T-cells *in vitro*.

Extensive amplification of these T-cells and their differentiation into effector cells requires signals through the IL-2R (Signal 3) or other T-cell proliferation receptors like IL-15 etc.



Helper T-cells and memory T-cells

Protocol for activating T-cells

Steps to measure T-cell activation in primary human CD4⁺ T-cells:

Step1: Isolate CD4⁺ T-cell from whole blood by negative selection with a Miltenyi CD4⁺ T-cell Isolation kit II (#130-091-155) (remove CD8 T-cells, δ/γ T-cells, B-cells, NK cells, dendritic cells, monocytes, granulocytes and erythroid cells).

Step 2: Thereafter, 5×10^4 T-cells/well were incubated with or without test compound on anti-CD3 and anti-CD28 coated wells for 24 hours to test IFN- γ in the supernatant, 48 hours for IL-2, respectively.

Step3: Then, 10 μ l supernatant were analyzed by HTRF either with Cisbio interferon- γ kit (#62IFNPEB) or Cisbio interleukin-2 kit (#64IL2PEB) according to the Cisbio protocol.

After the indicated incubation time plates were measured in a Pherastar/RubyStar and values analyzed for the ratio 665/620.

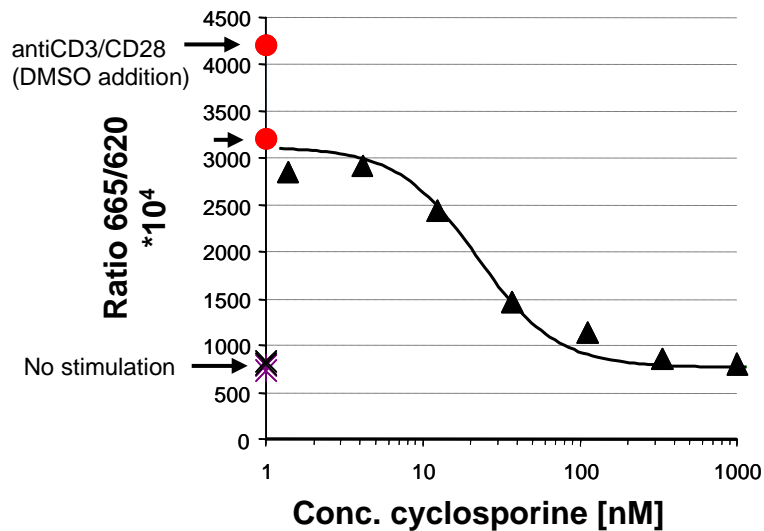
Activated primary human T-cell secrete IL-2 and IFN- γ after CD3/CD28 stimulation

Cyclosporine is a strongly immunosuppressive drug used mainly in the treatment of transplant patients. It blocks T-cell activation and the secretion of IFN- γ and IL-2.

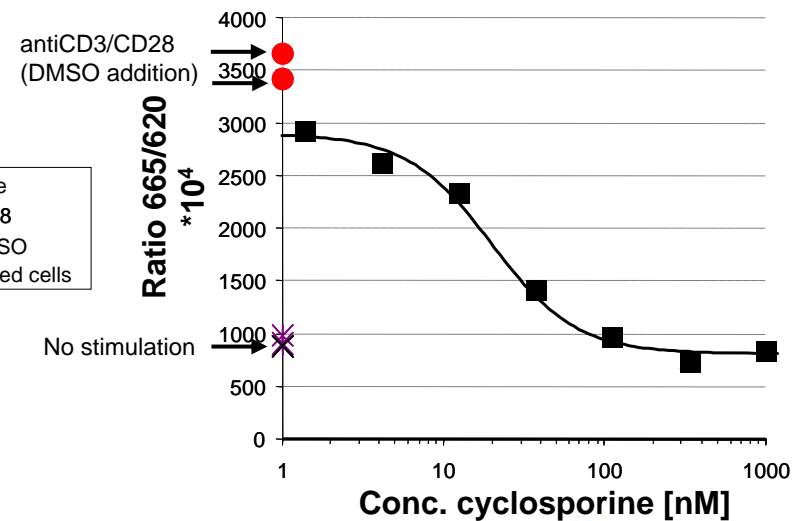
Both signature cytokines can be measured using the respective HTRF kits. IL-2 (IC_{50} 18 nM) and interferon- γ (IC_{50} 23 nM) are inhibited with the expected IC_{50} values for cyclosporine.

Both IC_{50} values match T-cell proliferation measured with 3H -thymidine (IC_{50} 40 nM)

Effect of cyclosporine on IL-2 secretion of human CD4⁺ T-cells stimulated with CD3/CD28

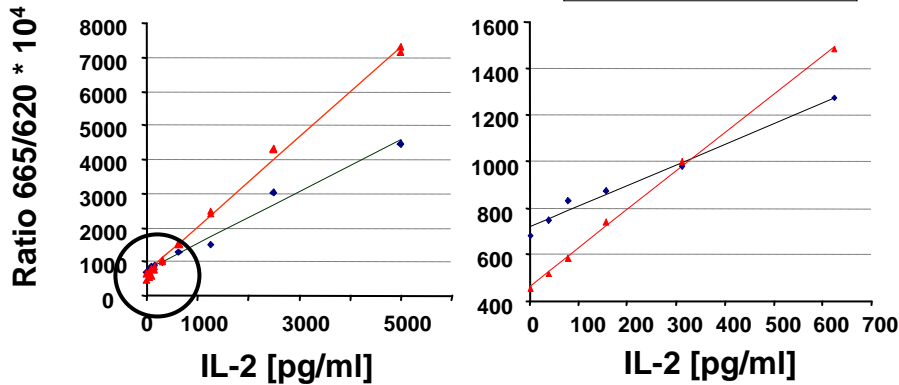


Effect of cyclosporine on IFN- γ secretion of human CD4⁺ T-cells stimulated with CD3/CD28



Sensitivity of HTRF assays for IL-2 and IFN- γ

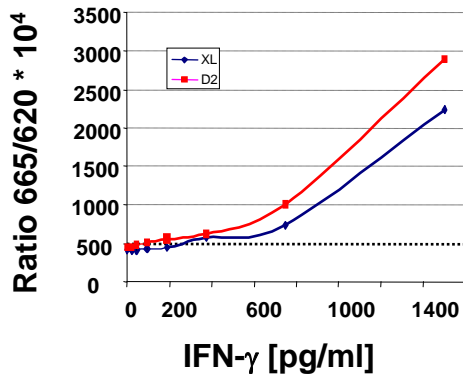
IL-2 standard curves



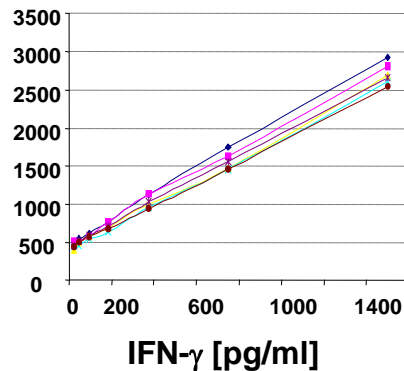
Both, the IL-2 and the interferon- γ assay have detection limits around 50 pg/ml and are therefore comparable to ELISA, which can detect lower than 10 pg/ml (not used in practice).

IFN- γ standard curves

IFN- γ Standard Novartis (frozen)



Cisbio: new reagents



Note for making “good” standard curves:

Avoid diluting and freezing standard cytokine solutions in order to make dilution curves.

Always use a highly concentrated stock solution, preferably snap frozen in liquid nitrogen.

Uses of HTRF IL-2- and interferon- γ kits

The Cisbio IL-2 and IFN- γ kits are routinely used to assess:

- Secondary cellular assay for targets interfering with T-cell activation, e.g. kinase targets, ion channel targets, transcription factors and others.
- T-cell activation and proliferation, including toxicity.
- SiRNA screening for new targets in T-cell activation but also induction of anergy.
- Characterization of T helper cell lineages (Th1 versus Th17).
- Study other interferon- γ producing cell types.

Comparison versus commercial ELISA kits:

- Sensitivity of IL-2 and IFN- γ is almost comparable to detection limits of ELISA (>40 pg/ml) for both kits.
- Kits are not sensitive to different cell culture media.

HTRF cytokine kits for T-cell lineage differentiation: The IL-17/ IFN- γ kits

Four T helper cell lineages are currently known: Th1, Th2, Th17 and T_{reg}

In the short time since the discovery of the Th17 helper cell lineage, the Th17 subset has been implicated in numerous autoimmune and inflammatory conditions including arthritis (Nakae et al.,2003; Murphy et al. 2003), multiple sclerosis (Cua et al.,2003; Langrish et al., 2005), psoriasis (Zheng et al.,2007), and inflammatory bowel disease (Hue et al., 2006).

IL-17 has profound pro-inflammatory effects and induces tissue damage during the course of many autoimmune diseases. Increased levels of IL-17A have been observed in rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis.

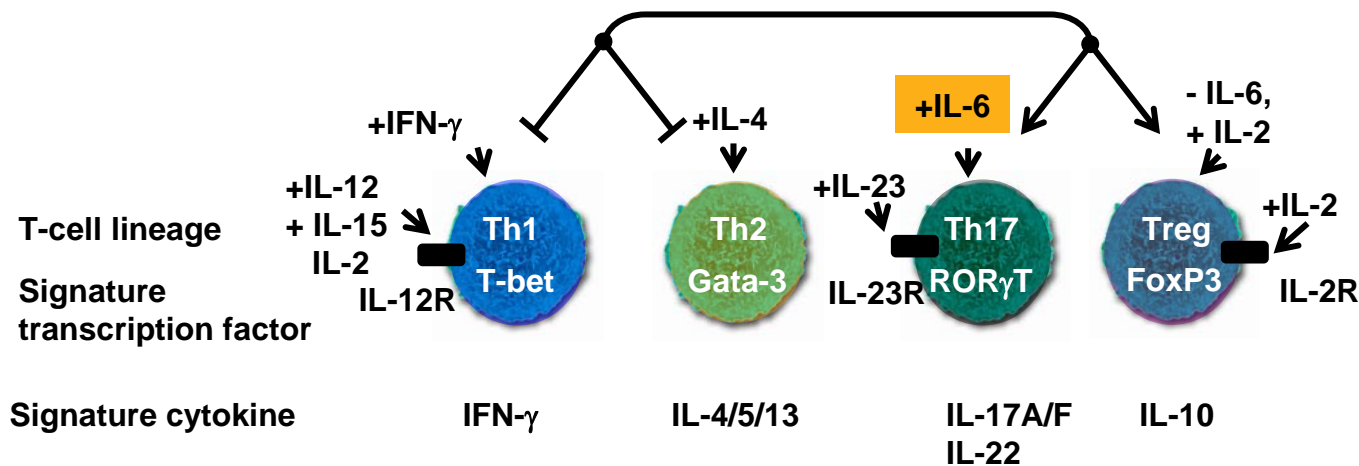
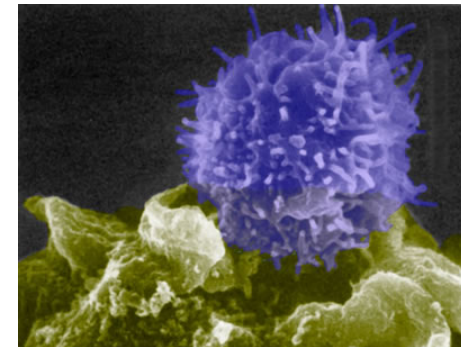
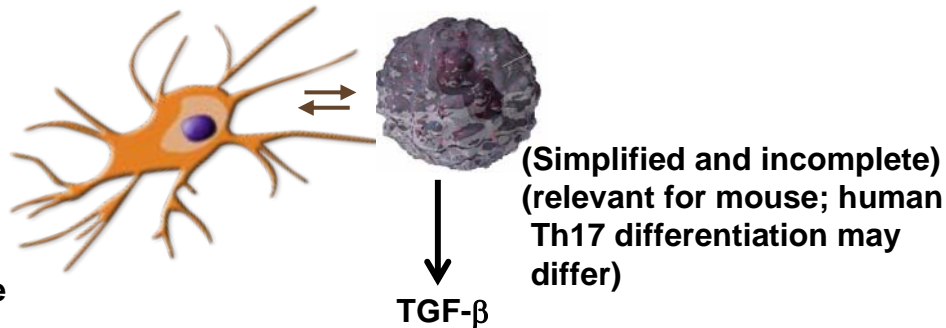
Therefore, Th-17 cells are of high therapeutic interest but the role of IL-17 in major human disease is currently much less well understood than that of TNF α or IFN- γ .

Comparative clinical trials of the efficacy of IL-17A blocking strategies versus IL-12/IL-23 are not yet available.

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Four different T-cell lineages shape immune response and control overreaction

Naïve T-cells receive instructions from dendritic cells and macrophages via cytokines and cell-cell contact; memory T-cells proliferate after TCR-stimulation



Understanding the factors (druggable targets) that influence the balance between Th17 and Treg cells may provide exciting new targets for inflammatory and autoimmune diseases

Intracellular pathogens	Parasitic worms	Extracellular bacteria	Counter regulation
Systemic pathology	Allergy and asthma	Autoimmunity Inflammation	

Protective role

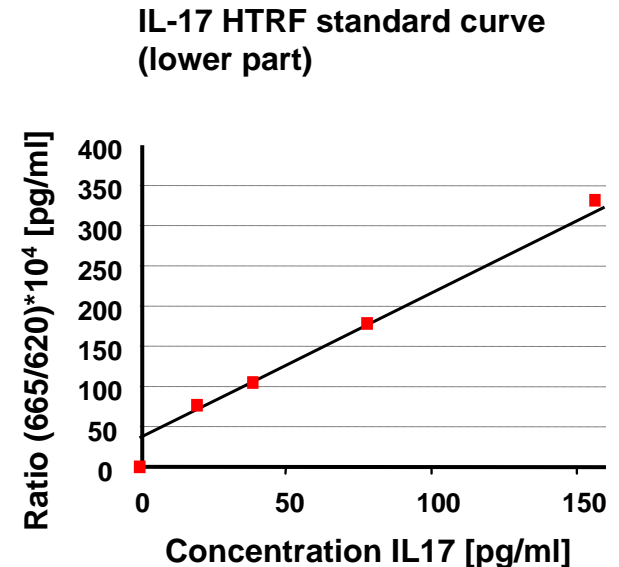
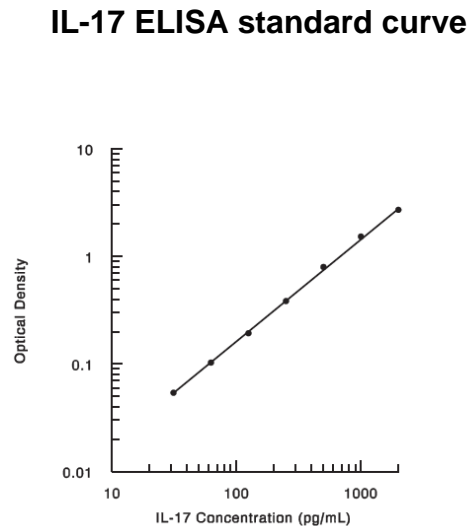
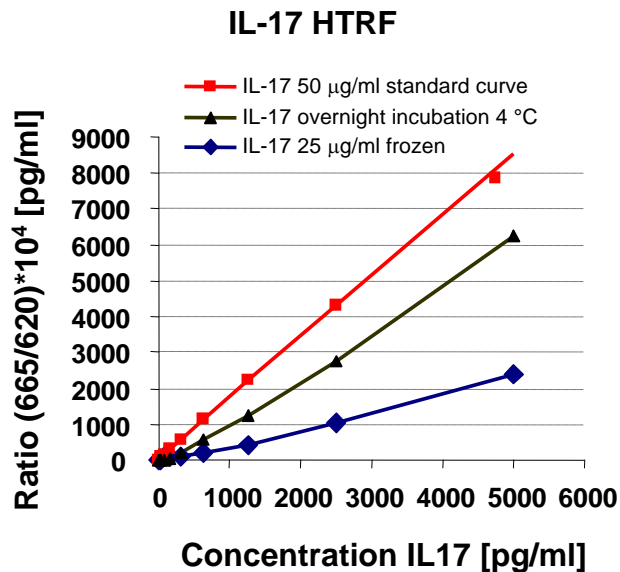
Harmful role

Standard curves for IL-17 using IL-17 HTRF kit

Comparing standard curves from commercial ELISA kits and HTRF show good sensitivity for the HTRF kit (~50 pg/ml).

Differentiated Th17 cells produce large quantities of IL-17A and IL-17F, that can be easily detected with the sensitivity of the kit. IL-17F not detected with HTRF.

HTRF standard curve is linear up to ~10 000 pg/ml.

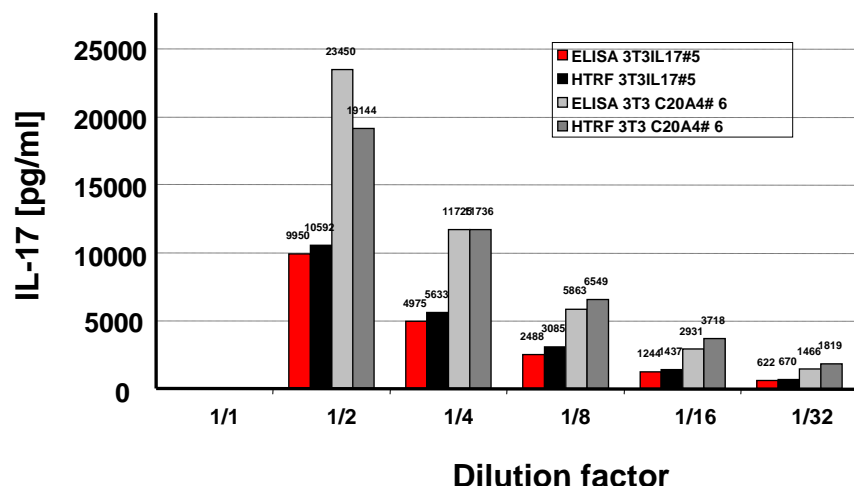


Measuring IL-17 from supernatant of IL-17 transfected 3T3 fibroblasts

The goal of this experiment was to identify IL-17 producing clones from retrovirally transfected 3T3 fibroblasts.

3T3 mouse fibroblasts were retrovirally transfected with IL-17 GFP construct and GFP-expressing clones were screened for IL-17 production.

Measure of IL17 secretion in supernatant culture medium from 3T3 IL17#5 +/- C20A4# 6 Cells in medium



Result:

IL-17 concentrations in supernatant are almost identical in ELISA and HTRF.

Some optimization required (0.05-0.1% final conc. Tween80) to reduce background.

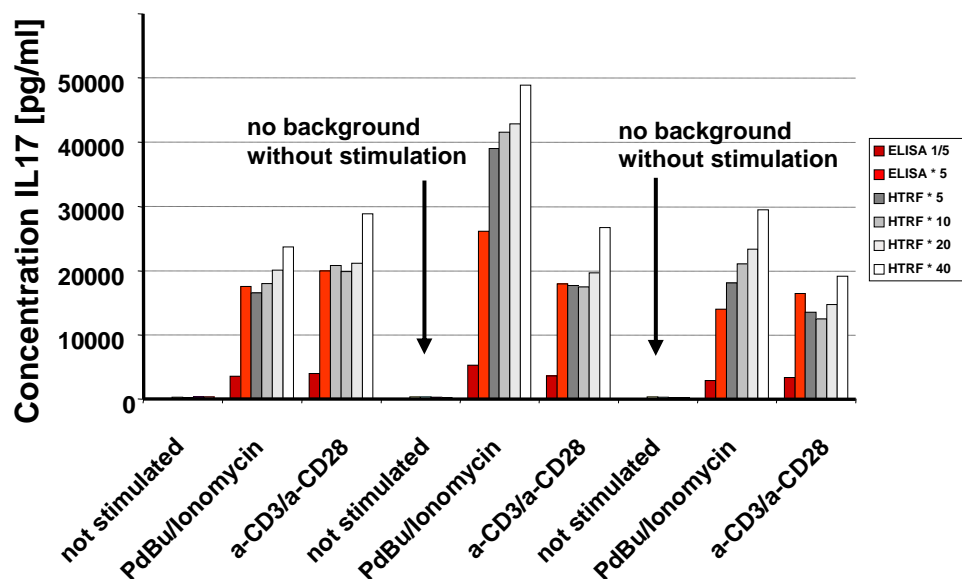
Measuring IL-17 in differentiated T-cell supernatants

Protocol for *in vitro* T-cell differentiation:

- 1) Isolate primary CD4 T-cells (Miltenyi kit) → 2) Culture under differentiating environment (TGF- β , IL-1 β , IL-6, antiIFN- γ , anti IL-4) (*)
- 3) **Activate** T-cell to trigger signature cytokine → 4) Readout IL-17/IFN- γ production

(*) after lineage commitment T-cell lineage are stable

Measure of IL17 secretion in supernatant culture medium from differentiated T-Cells.



Result:

IL-17 concentrations in supernatant have good correlation between ELISA and HTRF.

Working dilution recommended to be in 100-5000 pg/ml range.

Some optimization required to suppress background. (0.05% final conc. Tween80).

Uses of HTRF IL-17- and interferon- γ kits

1) Tracking Th17 differentiation and balance between Th17 and iTregs

Basic protocols for *in vitro* Th17 differentiation have been worked out (TGF- β , IL-6 plus anti IL-4/IFN- γ in mice, in humans IL-1 β and IL-23 seem to play a major role).

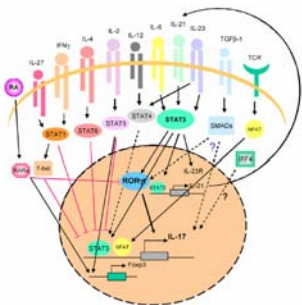
Currently, IL-6 seems to be a key factor that favors Th17 versus iTregs.

Nevertheless, many factors relevant *in vivo* are probably not yet identified:

Especially membrane bound proteins sitting on antigen presenting cells and instructing T-cell differentiation or costimulatory pathways in T-cells exist.

2) Screening for pathways that lead to IL-17 secretion and limiting the effector function of Th17

The signaling events that lead to IL-17 production are controlled by the orphan transcription factor ROR γ t and ROR α but detailed pathways are not yet available.



Complicated dependencies between signaling pathways have been described in the literature but not yet worked out systematically.

High throughput screening assays required to find druggable nodes?

Summary

- **The IL-2, IFN- γ and IL-17 kits are well suited to replace conventional ELISA kits for cytokine detection.**
- **Sensitivity of HTRF kits is quite close to ELISA and sufficient to allow assay design to be adapted to fit well into the standard curve.**
- **Nevertheless, all cytokine detection kits are “black box” cellular screens; smart immunological assay designs have to be set up to do target identification.**
- **We also use TNF α , IL-1 β , IL-8 and IL-6 kits from Cisbio to measure proinflammatory cytokines from supernatants besides T-cell specific cytokines.**

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