# Immunological laboratory testing in patients with tumours

**The success of an "immunostimulatory" therapy for patients with tumour diseases essentially depends on the following points:**

## 1. Is immunostimulation indicated?

- Immunostimulation must be indicated, i.e., it must be proven that those functional immune parameters are actually reduced. The stimulation of a functionally intact immune system is only helpful in exceptional cases. The current immunocompetence can only be evaluated through functional tests (LTT immune function, NK cell cytotoxicity test) and only with that it can successfully be controlled afterwards.
- It must be excluded that a pre-existing immune activation is not the cause of a reduced lymphocyte or NK cell function. In the cellular immune profile, therefore, the $HLADR^+$ T cells should not be substantially elevated and the CD4/CD8 ratio should not be lower than 0.8.
- It must be proven that the patient has sufficient immune cells for stimulation (espcially $CD4^+$ helper cells). 250 $CD4^+$ cells/µl are indicated as the limit. Otherwise, an immune-restorative therapy is preferable to immunostimulatory measures (trace elements, vitamins, thymus).

## 2. Is the immune system moving in the right direction during therapy?

During immunostimulatory therapy (e.g. with preparations containing mistletoe, organs or microorganisms), the following laboratory parameters can be consulted to evaluate progress and prognosis. Following questions can be addressed:

- **Was a TH1 polarisation achieved?**

  The analysis is performed in the TH1/TH2 profile with measurement of Interferon-gamma (IFN-γ = TH1) and Interleukin-4 (IL-4 = TH2) after stimulation of the patient's T lymphocytes. Basal T cell cytokine levels (in the blood) are not informative, because even in severe imbalances these cytokines IFN-γ / IL-4 are released into the blood in only insufficient quantities. The goal of immunostimulatory therapy is always a TH1 polarisation (i.e. IFN-γ ↑, IL-4 ↓). The IFN-γ-producing TH1 cells are the most important effector cells that contribute to eliminating "abnormal" cells.

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| Test | result | unit | reference range |
|---|---|---|---|
| **TH1/TH2 - balance** | | | |
| The cytokine concentrations after 24 hours are indicated. Stimulation with ConA/SEB. | | | |
| IFN-γ (TH1) | 463 | pg/ml | 374 - 1660 |
| IL-4 (TH2) | **839** | pg/ml | 40 - 198 |
| TH1 / TH2 ratio | **0,55** | | 3,5 - 11 |
| The result shows a clear TH1/TH2 imbalance associated with the TH2 immune response. | | | |

**Fig. 1** Unfavourable TH2 predominance in a patient with metastatic rectal cancer before therapy. The goal of immunostimulatory therapy is an increase of IFN-γ with a decrease in IL-4.

- **Does the proportion of immunosuppressive regulatory T cells (Treg) remain low**?

  The regulatory T cells ($T_{reg}$) are an important sub-group of the CD4 cells (normal approx. 4-10 %). They play a central role in the maintenance of immunotolerance, which is counterproductive in tumour illnesses. Regulatory T cells inhibit the effector functions of cytotoxic T cells, NK cells and other immune cells against tumour antigens and thus support tumour growth. A direct correlation between the number of $T_{reg}$ cells and the stage of the tumour could be detected. Advanced tumour stages showed an increased infiltration of the tissue with Tregs. In addition, an inverse correlation between the number of $T_{regs}$ in the tumour tissue and survival rate could be pointed out.

  $T_{reg}$ cells are well suited as progress markers in immunomodulating therapies. It is prognostically favourable if they do **not** rise. Regulatory T cells are phenotypically identified by flow cytometry based on the cell surface marker constellation $CD4^+CD25^{++}CD127^{low}$.

  In addition, the surface molecule CD39 is identified on $T_{reg}$ cells. CD39 is a peripheral membrane protein (ectoenzyme), which causes the transformation of ATP and ADP into AMP. AMP is converted extracellularly into adenosine, which acts as an immunosuppressant. The detection of the $CD39^+$ $T_{reg}$ fraction can thus give a clue about the current immunosuppressive capacity of the $T_{reg}$ cells.



IMD Berlin MVZ

Nicolaistraße 22
12247 Berlin (Steglitz)

Tel. +49 (0)30 77001-220
Fax +49 (0)30 77001-236

Info@IMD-Berlin.de
IMD-Berlin.de

- **Do the CD8 cells with cytotoxic effector capacity increase?**

  The term CD8 suppressor cells which is used incorrectly even today for the entire CD8 population is out-of-date. The "CD28 Status" differentiates the CD8 lymphocytes in cells with cytotoxic properties and those that actually perform a suppressive (immunosuppressive) function. As a matter of course the goal of immunostimulatory therapy is to increase the proportion of cytotoxic i.e. CD28-positive CD8 cells.

- **Do the activated natural killer cells increase?**

  It is prognostically favourable, that during immunostimulatory therapy activation markers such as CD25 (receptor for interleukin-2) increase on natural killer cells. However, this test does not replace analysis of the NK cell function.

- **What is the patient's residual thymic function like?**

  The surface marker CD31 defines a subpopulation of the naive $CD4^+$ helper cells, which only recently left the thymus. These are referred to as "recent thymic emigrants – RTE cells". The proportion of the $CD31^+$ naive CD4 cells in the blood is thus a measure of the residual thymic function. A decreased thymic function is exhibited in a reduced "ability to replenish" virgin T lymphocytes if there is an intensified "consumption" due to infections or after chemotherapy or radiotherapy. Therefore, the test is useful in the event of therapies that are immunologically damaging to assess the regeneration capacity and possible planning of early intervention (immune restoration marker).

**The parameters mentioned are included in the quantitative immune profile "Immunocompetence Tumour" in addition to the recognised standard analyses such as CD4, CD8, NK and B cells and activated T cells.**

## Which diagnostic procedure is recommended?

Before immunostimulatory therapy

1. LTT immune function + NK cell cytotoxicity test (as an indication and a starting point)
2. Quantitative immune profile "Immunocompetence Tumour "
3. TH1/TH2 profile (as initial value)

The repetition of the analyses 6-8 weeks after beginning immunostimulation answers the following questions:

1. Did the function of the T lymphocytes and NK cells improve?
2. Is there a shifts in the cell markers which point toward a sustained improvement in the immunological situation (esp. $T_{reg}$ cells and a portion of $CD39^+$ Treg cells, $CD28^+$ cytotoxic T cells)?
3. Could a TH1 polarisation be achieved (IFN-γ increase and/or IL-4 decrease)?

Subsequently, while check-ups 4-8 months apart are recommended regardless of the clinical situation, one may be limited to the pathological findings in the initial diagnosis.

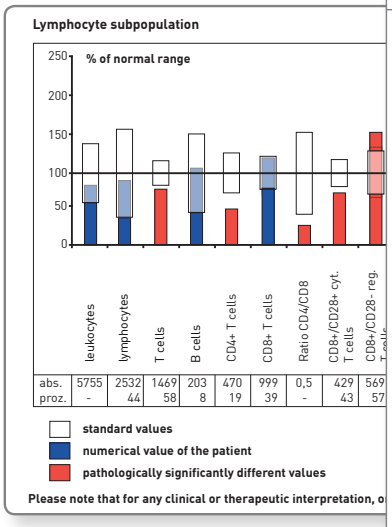


**Fig. 2** The quantitative immune profile "Immuno-competence Tumour" was conducted as the initial state of a 58-year-old female patient with breast cancer. Due to the fact that regulatory T cells like $CD8^+/CD28^+$ as well as the percentage of $CD39^+$ Tregs ($CD4^+/CD25^{++}/CD127^{low}$) are increased an immunostimulatory therapy should be carefully monitored. Regulatory cell subsets should not increase under immunostimulatory intervention.

The result is always displayed numerically and graphically.

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| | | standard values | | standard values |
|---|---|---|---|---|
| Leukocytes | **5755** /µl | 3900 - 10200 | | |
| Lymphocytes | **2532** /µl | 1100 - 4500 | **44** % | 20 - 44 |
| Monocytes | **576** /µl | 100 - 900 | **10** % | 2 - 9,5 |
| Granulocytes | **2647** /µl | 2400 - 7400 | **46** % | 42 - 75 |
| **Immune competence** | | | | |
| T cells | **1469** /µl | 920 - 2580 | **58** % | 61 - 84 |
| B cells | **203** /µl | 120 - 630 | **8** % | 7 - 21 |
| NK cells | **861** /µl | 210 - 740 | **34** % | 10 - 30 |
| CD4+ T helper cells | **470** /µl | 550 - 1460 | **19** % | 32 - 60 |
| CD8+ T cells | **999** /µl | 280 - 930 | **39** % | 23 - 40 |
| naive T cells (CD45RA+) | **279** /µl | 300 - 1200 | **19** % | 30 - 63 |
| Thymic function (CD31+) | | | **88** % | > 49 |
| **Immune activation** | | | | |
| CD4/CD8 ratio | **0,47** | 1 - 3 | | |
| acitvated T cells (HLA-DR+) | **206** /µl | < 230 | **8** % | < 11 |
| pre-activated T cells (CD25+) | **177** /µl | < 230 | **7** % | < 18 |
| memory T cells (CD45RO+) | **1190** /µl | 300 - 1300 | **81** % | 37 - 70 |
| CD8+/CD28+ (cytotoxic) | **429** /µl | 238 - 448 | **43** % | 49 - 73 |
| activated NK cells | **23** /µl | < 40 | **2,7** % | < 17 |
| CD4+/CD8+ T cells | | | **0,7** % | < 5 |
| **Immune tolerance** | | | | |
| Treg (CD4+/CD25++/CD127low) | **22** /µl | 35 - 120 | **4,7** % | 4 - 10 |
| subset CD39+ Treg | | | **64** % | < 54 |
| CD8+/CD28- (regulatory) | **569** /µl | 100 - 370 | **57** % | 26 - 51 |
| CD8/CD28 ratio | **0,75** /µl | 1 - 2,8 | | |

**Immunocompetence:** Intact – only slightly reduced CD4 cells
**Thymus reserve:** Within the age-specific reference range
**Immune activation:** Moderate signs – slightly elevated CD8- and NK- cells as well as an increased proportion of memory T cells (indication of chronic immune activation)
**Immune tolerance:** Tendency towards an increase – increased regulatory (CD8+/CD28-) T cells and increased proportion of CD39+ Tregs (CD4+/CD25++/CD127low), which may indicate functional immunosuppression.

**Explanatory representation of a very good progress finding after alternating therapy with mistletoe and a thymic preparation for a 54-year-old female patient with breast cancer.**

## 1. LTT Immune Function

### Preliminary findings

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**Lymphocyte transformation test for immune function**

| Cellular immune function | SI |
|---|---|
| Influenza | 6,3 |
| Tetanus toxoid | 6,2 |
| Cytomegalovirus | 9,3 |
| Varizella zoster | 2,5 |
| Candida | 9,2 |
| Streptokokken | 7,6 |

**Mean function index:** 6,8

The mean function index is calculated from the mean of the 6 antigen-stimulated indices (see fields below), which is better than the individual parameters for assessing and monitoring the immune function.

**Explanation of the test me**

The LTT tests the antigen-s lymphocytes. It does this (induced proliferation) th are a component of wid vaccines.
Because the latter only le an intact immune functio current immunocompete The mean function index effective immunostimular

| Normal values: | | |
|---|---|---|
| | >15 | good immune function |
| | 10 - 15 | satisfactory immune function |
| | <10 | reduced immune function |
| | < 7 | significantly reduced immune function |

Blank value (negative c
Mitogen control (PWM)

**Results:**
Evidence of a reduced cellular immune function recognisable by the mean fu
In lights of this finding an immunostimulatory therapy would be indicated at the
Independently of how the immunstimulation is conducted carried out the succ
checked about 6-8 weeks after the start of therapy with the LTT. In the positive c
notably. The target value should be at least 15.

### Medical finding at 6-week follow-up

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**medical report**

**Lymphocyte transformation test for immune function**

| Cellular immune function | SI |
|---|---|
| Influenza | 14,5 |
| Tetanus toxoid | 14,3 |
| Cytomegalovirus | 18,0 |
| Varizella zoster | 7,7 |
| Candida | 17,9 |
| Streptokokken | 13,6 |

**Mean function index:** 14,3

The mean function index is calculated from the mean of the 6 antigen-stimulated indices (see fields below), which is better than the individual parameters for assessing and monitoring the immune function.

**Explanation of the test methodology:**

The LTT tests the antigen-specific reactivity of the T helper lymphocytes. It does this by measuring the ability to activate (induced proliferation) the lymphocytes. Antigenic stimulants are a component of widespread infectious pathogens or vaccines.
Because the latter only lead to a significant cell proliferation with an intact immune function, inferences can be made about the current immunocompetence using the mean function index. The mean function index should increase in response to an effective immunostimulant.

| Normal values: | | |
|---|---|---|
| | >15 | good immune function |
| | 10 - 15 | satisfactory immune function |
| | <10 | reduced immune function |
| | < 7 | significantly reduced immune function |

| | |
|---|---|
| Blank value (negative control) | 1404 (normal value < 4000 cpm) |
| Mitogen control (PWM) | 7046 (normal value > 20000 cpm) |

**Results:**
Compared to the preliminary findings, the T cell immunocompetence improved, shown by the rise in the mean function index from 6,8 to14,3.
The improvement in the function of the T helper cells is likely due to the increase in the function of the natural killer cells and the now normalised IFNg/IL-4 ratio (see separate result).
We recommend a continuation of the therapy and if necessary a check-up in 4-6 weeks.

**Fig. 3** The LTT immune function permits the analysis of the function of lymphocytes as well as indirectly monocytes and dendritic cells. The test is based on the principle of lymphocyte stimulation by memory antigens (recall antigens), against which should induce a strong immune response in intact cellular immunocompetence. When the assay is performed, these antigens are presented by monocytes and dendritic cells to T helper cells. These are activated in correlation with the current immune function and undergo cell division. The obligatory DNA synthesis is quantitatively detected.

## 2. NK cell cytotoxicity test and TH1/TH2-Profile

### Preliminary findings

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| Test | Result | Unit | Reference-range |
|---|---|---|---|
| NK cell cytotoxicity test | | | |
| During the test, the rate of K562 tumour cells is analysed, which are kiled by the natural killer cells (NK cells), isolated from the patient´s heparin blood. The tumour cells apoptasis rate identifes the patient´s current NK cells function. The value for the apoptosis rate during IL-2 stimulation indicates the additional stimulatability of the NK cells. | | | |
| Tumour cell apoptosis rate | 15,8 | | |
| Apoptosis-rate-IL2-stimulated | 22,9 | | |
| Interpretation | | | |
| Reduced function of the natural killer cells. | | | |
| **TH1/TH2 balance** | | | |
| The cytokine concentration ist determined after 24 hours of stimulation with ConA/SEB. | | | |
| IFN-g (TH1) | 125 | | |
| IL-4 (TH2) | 366 | | |
| TH1/TH2 Ratio | 0,34 | | |
| The result shows a clear TH1/TH2 imbalance associated with an increased TH2 immune pher | | | |

### Medical finding at 6-week follow-up

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| Test | Result | Unit | Reference-range |
|---|---|---|---|
| NK cell cytotoxicity test | | | |
| During the test, the rate of K562 tumour cells is analysed, which are kiled by the natural killer cells (NK cells), isolated from the patient´s heparin blood. The tumour cells apoptasis rate identifes the patient´s current NK cells function. The value for the apoptosis rate during IL-2 stimulation indicates the additional stimulatability of the NK cells. | | | |
| Tumour cell apoptosis rate | 43,7 | % | > 17 |
| Apoptosis-rate-IL2-stimulated | 71,8 | % | |
| Interpretation | | | |
| Compared to preliminary findings significant increase of NK cell function. | | | |
| **TH1/TH2 balance** | | | |
| The cytokine concentration is determined after 24 hours of stimulation with ConA/SEB. | | | |
| IFN-g (TH1) | 677 | pg/ml | 374 - 1660 |
| IL-4 (TH2) | 123 | pg/ml | 40 - 198 |
| TH1/TH2 Ratio | 5,50 | | 3,5 - 11 |
| Compared to preliminary findings significant increase of TH1 immune cells (IFN-γ) and decrease of TH2 cell fraction (IL-4). | | | |

**Fig. 4** For the NK cell cytotoxicity test, a tumour cell line is labelled with the membrane dye Calcein. Subsequently, the patient's NK cells are added under standardised conditions. During the lysis process induced by the NK cells, the Calcein dye is released from the tumour cells and then quantitatively determined in cell culture supernatant. By parallel testing of standardised controls, the percentage of destroyed tumour cells can be determined.
To examine the TH1/TH2 balance heparinised blood is incubated with non-specific stimulants (Concanavalin A and SEB) for 24 h. Subsequently, the cytokines released into the supernatant are determined by multiplex cytokine assay.

## 3. Immune profile "Immunocompetence Tumour"

### Preliminary findings

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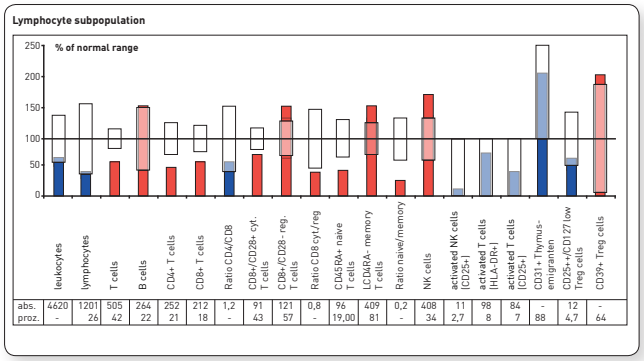
| | | standard values | | standard values |
|---|---|---|---|---|
| Leukocytes | 4620 /µl | 3900 - 10200 | | |
| Lymphocytes | 1201 /µl | 1100 - 4500 | 26 % | 20 - 44 |
| Monocytes | 416 /µl | 100 - 900 | 9 % | 2 - 9,5 |
| Granulocytes | 3003 /µl | 2400 - 7400 | 65 % | 42 - 75 |
| **Immune competence** | | | | |
| T cells | 505 /µl | 920 - 2580 | 42 % | 61 - 84 |
| B cells | 264 /µl | 120 - 630 | 22 % | 7 - 21 |
| NK cells | 408 /µl | 210 - 740 | 34 % | 10 - 30 |
| CD4+ T helper cells | 267 /µl | 550 - 1460 | 22 % | 32 - 60 |
| CD8+ T cells | 227 /µl | 280 - 930 | 19 % | 23 - 40 |
| naive T cells (CD45RA+) | 96 /µl | 300 - 1200 | 19 % | 30 - 63 |
| Thymus function (CD31+) | | | 88 % | > 54 |
| **Immune activation** | | | | |
| CD4/CD8 ratio | 1,18 | 1 - 3 | | |
| acitvated T cells (HLA-DR+) | 96 /µl | < 230 | 8 % | < 11 |
| pre-activated T cells (CD25+) | 84 /µl | < 230 | 7 % | < 18 |
| memory T cells (CD45RO+) | 409 /µl | 300 - 1300 | 81 % | 37 - 70 |
| CD8+/CD28+ (cytotoxic) | 98 /µl | 238 - 448 | 43 % | 49 - 73 |
| activated NK cells | 11 /µl | < 40 | 2,7 % | < 17 |
| CD4+/CD8+ T cells | | | 0,4 % | < 5 |
| **Immune tolerance** | | | | |
| Treg (CD4+/CD25++/CD127low) | 13 /µl | 35 - 120 | 4,7 % | 4 - 10 |
| subset CD39+ Treg | | | 64 % | < 54 |
| CD8+/CD28- (regulatory) | 129 /µl | 100 - 370 | 57 % | 26 - 51 |
| CD8/CD28 ratio | 0,75 /µl | 1 - 2,8 | | |

**Immunocompetence:** Reduced – CD4 and CD8 cells are reduced
**Thymus reserve:** Within the age-specific reference range
**Immune activation:** Subtle indications – increased proportion of memory T cells (indication of chronic immune activation)
**Immune tolerance:** Tendency towards increased – regulatory (CD8+/CD28-) T cells increased as well as a higher proportion of CD39+ Treg (CD4+/CD25++/CD127low) which may indicate functional immunosuppression.




### Medical finding at 6-week follow-up

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| | | standard values | | standard values |
|---|---|---|---|---|
| Leukocytes | 5800 /µl | 3900 - 10200 | | |
| Lymphocytes | 1160 /µl | 1100 - 4500 | 20 % | 20 - 44 |
| Monocytes | 348 /µl | 100 - 900 | 6 % | 2 - 9,5 |
| Granulocytes | 4292 /µl | 2400 - 7400 | 74 % | 42 - 75 |
| **Immune competence** | | | | |
| T cells | 789 /µl | 920 - 2580 | 68 % | 61 - 84 |
| B cells | 151 /µl | 120 - 630 | 13 % | 7 - 21 |
| NK cells | 220 /µl | 210 - 740 | 19 % | 10 - 30 |
| CD4+ T helper cells | 497 /µl | 550 - 1460 | 43 % | 32 - 60 |
| CD8+ T cells | 260 /µl | 280 - 930 | 22 % | 23 - 40 |
| naive T cells (CD45RA+) | 245 /µl | 300 - 1200 | 31 % | 30 - 63 |
| Thymus function (CD31+) | | | 79 % | > 54 |
| **Immune activation** | | | | |
| CD4/CD8 ratio | 1,91 | 1 - 3 | | |
| acitvated T cells (HLA-DR+) | 104 /µl | < 230 | 9 % | < 11 |
| pre-activated T cells (CD25+) | 151 /µl | < 230 | 13 % | < 18 |
| memory T cells (CD45RO+) | 544 /µl | 300 - 1300 | 69 % | 37 - 70 |
| CD8+/CD28+ (cytotoxic) | 164 /µl | 238 - 448 | 63 % | 49 - 73 |
| activated NK cells | 6 /µl | < 40 | 2,7 % | < 17 |
| CD4+/CD8+ T cells | | | 0,1 % | < 5 |
| **Immune tolerance** | | | | |
| Treg (CD4+/CD25++/CD127low) | 27 /µl | 35 - 120 | 5,4 % | 4 - 10 |
| subset CD39+ Treg | | | 51 % | < 54 |
| CD8+/CD28- (regulatory) | 96 /µl | 100 - 370 | 37 % | 26 - 51 |
| CD8/CD28 ratio | 1,7 /µl | 1 - 2,8 | | |

Compared to the preliminary finding, a tendency towards an increase in CD4 cells is observed.
The fraction of cytotoxic (CD8+/CD28+) T cells has increased.
Drop in the CD39+ Tregs with an almost unchanged overall proportion of CD4+/CD25++/CD127low Treg cells as well as a decline in the number of regulatory (CD8+/CD28-) T cells.
In light of this finding, there is no contraindication to continue therapy.

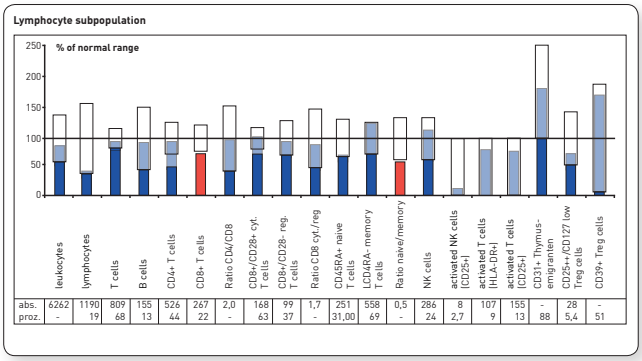


**Fig. 5** The immune cells are differentiated based on the binding of fluorescence-marked monoclonal cell-specific antibodies and subsequent flow cytometry analysis. The graphical representation serves to better recognise deviations in cell distributions.

## Material

**LTT Immune Function:**
20 ml heparin blood + 5 ml whole blood (serum)

**NK cell cytotoxicity test:**
10 ml heparin blood

**Quantitative immune profile "Immunocompetence Tumour "**
2 ml EDTA blood

**TH1/TH2-Profile:**
5 ml heparin blood

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## Literature


- AM Gallimore et al. Positive and negative influences of regulatory cells on tumour immunity. Oncogene 2008; 27:5586-93
- Kohler S, Thiel A. Life after the thymus - CD31+ and CD31- human naive CD4+ T-cell subsets. Blood. 2008; 26
- Vrisekoop N, van Gent R, de Boer AB, Otto SA, Borleffs JC, Steingrover R, Prins JM, Kuijpers TW, Wolfs TF, Geelen SP, Vulto I, Lansdorp P, Tesselaar K, Borghans JA, Miedema F.: Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging. J Immunol. 2008; 15;181:1573-81
- Kilpatrick RD, Rickabaugh T, Hultin LE, Hultin P, Hausner MA, Detels R, Phair J, Jamieson BD. Homeostasis of the naive CD4+ T cell compartment during aging. J Immunol. 2008 1;180:1499-507.
- Sun X, Wu Y, Gao W, Enjyoji K, Csizmadia E, Müller CE, Murakami T, Robson SC.CD39/ENTPD1 expression by CD4+Foxp3+ regulatory T cells promotes hepatic metastatic tumor growth in mice.