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Immunopathogenic and regulatory leukocyte subsets in juvenile idiopathic arthritis

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INTRODUCTION

JIA definition and classification

Juvenile idiopathic arthritis (JIA) is the most common pediatric rheumatologic disease and an important cause of short- and long-term disability in children. It is defined as the occurrence of arthritis for a period of more than six weeks in patients under age 16. Children present with joint swelling, pain, morning stiffness or simply avoid using the affected joints in their daily lives.

JIA is a very heterogenous disease and can be categorised in different subtypes. There have been numerous classifications trying to form homogenous mutually exclusive groups based on clinical presentation, laboratory features and exclusion criteria. The International League of Association for Rheumatology (ILAR) developed the classification currently in use (Petty et al., 2004). According to this classification JIA can be divided into seven subtypes: oligoarthritis (OA), rheumatoid factor (RF) positive polyarthritis (PA), RF negative polyarthritis, enthesitis-related arthritis (EA), psoriatic arthritis (PS), systemic JIA (sJIA) and undifferentiated arthritis. The distinction is made based on the number of affected joints, the location of inflammation, associations with specific genotypes or other chronic diseases, and the expansion of inflammation (Table 1). Patients with oligoarthritis have less than four inflamed joints. Based on the course of disease during the first six months, OA is subdivided into a persistent and an extended form. In persistent OA no more than four joints will be affected, whereas in extended OA, more than four joints show signs of arthritis with time. Anti-nuclear antibodies (ANAs) are found in the serum of more than half of the patients with OA. The presence of ANAs indicates loss of tolerance against nuclear autoantigens. However, the distinct autoantigens of these ANAs have not been identified yet. In all JIA subgroups the presence of ANAs is associated with a higher risk to develop uveitis (Heiligenhaus et al., 2007) (Saurenmann et al., 2010) and thus, ANA testing is part of the initial diagnostic work-up in JIA.

The subset of polyarthritis consist of patients that present with more than four affected joints when first diagnosed. Within polyarthritis the presence of RF distinguishes two further subgroups: rheumatoid factor positive and negative PA. Rheumatoid factors are autoantibodies that recognize the Fc region of IgG antibodies. In adult rheumatoid arthritis they are used for diagnostic purposes, since

80% of patients are positive. Over 70% of patients with RF+ PA JIA also have antibodies against cyclic citrullinated peptides (CCP) (Tebo et al., 2012). Anti- CCP antibodies have a high specificity in adult rheumatoid arthritis. However in JIA, most patients with anti- CCP antibodies are found in the subset of RF+ PA, while in patients of all other disease entities CCP antibodies are present in only 6%.

Table 1 Classification of different JIA subtypes according to several criteria.
m= male, f= female, ESR= Erythrocyte Sedimentation Rate, Hb= Hemoglobin.
*10% of all JIA patients belong to the undifferentiated group

Criteria	Systemic JIA	Oligoarthritis	Polyarthritis RF-	Polyarthritis RF+	Psoriatic arthiris	Enthesitis-related arthritis
Frequency*	5%	30%	20%	5%	5%	25%
Affected joints	Mostly polyarticular arthritis	Asymmetric arthritis: <4 joint in the first 6 month, mostly big joints	Aymmetric arthritis: >4 joints in the first 6 month, small and big joints, tend to affect fewer joints than in RF+	Aymmetric arthritis: >4 joints in the first 6 month, small and big joints	Typically in fingers or toes middle and end joints and dactolyitis	Asymmetric arthritis: mostly big joints
HLA involvement	DRB1, DQA1	A2, DRB1, DQA1, DQB1	A2, DRB1, DQA1, DPB1	DRB1, DQA1, DQB1	DRB1, DQA1	B27, DRB1, DQA1, DQB1
Age at onset	2-5 years	< 5 years	1-4 and 6-12 years	>8-10 years	7-10 years	9-12 years
Sex bias	f = m	m << f	m < f	m << f	m < f	m >> f
Extra articular feature	ESR ↑↑↑ Leukocytes ↑↑ Platelets ↑	ESR n/↑	ESR ↑↑↑ Hb ↓	ESR ↑↑↑ Hb ↓	ESR n/↑ Hb ↓	
	intermitted fever, hepato-splenomegaly, serositis, adenitis, rash	50% iridocyclitis especially in ANA+ patients,	subfebril condition, tiredness	subfebril condition, tiredness	70-80 % nail pathologies 10% uveitis	enthesitis, back pain, acute uveitis
Auto-antibodies	Mostly ANA- mostly RF- 13% anti-CCP+	60-80% ANA+ mostly RF- 10% anti-CCP+	50-80% ANA+ 100% RF- 8% anti-CCP+	50% ANA+ 100% RF+ 70% anti-CCP+	40-60% ANA+	rarely ANA+ 4% anti-CCP+

Enthesitis-related arthritis involves arthritis and enthesitis, meaning that patients complain of tenderness at the insertion of the ligaments or the joint capsule. It is the only subtype that has a higher prevalence in males and associated with HLA-B27. Children with psoriatic arthritis present with joint swelling and symptoms of psoriasis,

including typical skin lesion and non-dermal features such as nail pitting or dactylitis. This group also includes JIA patients that have a first-degree relative diagnosed with psoriasis. Systemic JIA is the most severe form and has very distinct features: Children suffer from fever, skin rash, lymph node enlargement, hepato-/splenomegaly and serositis. All these symptoms reflect the systemic involvement of this subtype (Petty et al., 2004). The unique presentation of symptoms gives evidence that the pathogenesis of systemic JIA is different to all other subtypes.

Epidemiology

Incidence and prevalence of JIA vary in a wide range depending on which studies are considered. One of the main reasons for this is the lack of clear diagnostic criteria and the heterogeneity of this disease. Additionally, the differential diagnosis of joint swellings includes many diseases such as reactive arthritis, connective tissue diseases, vasculitis, inflammatory bowl diseases and malignant bone tumors so that JIA is usually a diagnose of exclusion. In a comprehensive study of Manners et al. the prevalence of JIA ranges from 0,07 to 4,01 per 1000 children and they report incidences from 0,008 to 0,226 per 1000 children worldwide (Manners and Bower, 2002). These variations might be due to different standards of living and health care resources in developing countries, where the level of under diagnosed children is quite high. The German Society for paediatric rheumatology states that in Germany the incidence of JIA is 0,1 per 1000 children (Rheumatologie, 2008).

A Canadian epidemiologic study calculated the relative risk of children from different ethnic groups to develop JIA. Children of European background had the highest risk to develop any form of JIA, except the subtype of PA RF+ JIA. Children of African or Indian heritage had the highest risks to develop PA RF+ JIA while for all other subtypes the risk was significantly decreased. In Asia, overall prevalence of JIA is lower than in Europe or North America, while enthesitis-related JIA is overrepresented (Saurenmann et al., 2007). The differences in ethnic JIA subtype distribution indicate that there is a genetic predisposition to develop disease.

Etiopathogenesis

Although the etiopathogenesis of juvenile arthritis is not well defined, JIA is considered a multifactorial autoimmune disease, affecting individuals with a genetic susceptibility and involving at the same time environmental factors that trigger onset and exacerbation of disease.

Genetic susceptibility

A variety of genes are associated with the development of JIA (Schork, 1997). As it is the case in many autoimmune diseases, the highest association is found within the genes of the major compatibility complex, in humans named human leukocyte antigen (HLA). There are two types of HLA molecules, HLA class I and HLA class II. HLA class I molecules (HLA-A, -B, -C) are used by all nucleated cells to present endogenous peptides, whereas class II molecules (HLA-DR, -DQ, -DR) present extracellular peptides that have been taken up and processed by antigen presenting cells. HLA genes are associated with different subtypes of JIA: while certain HLA-A2 class I alleles are often in patients with an early onset of disease, HLA-B27 is strongly associated with EA (Prahalad and Glass, 2008). However, twin studies have shown that only 17% of the genetic risk to develop JIA comes from the HLA gene locus (Prahalad et al., 2000) suggesting that other non-HLA genes also contribute to the susceptibility of an individual to develop arthritis. One candidate is the IL2RA/CD25, encoding for the alpha chain of the interleukin-2 receptor. Recent studies show that single nucleotide polymorphisms in the IL2RA/CD25 gene locus, previously found associated with other autoimmune diseases such as multiple sclerosis (MS) and diabetes mellitus type 1, are also found in JIA (Hinks et al., 2009). Additionally, genes such as PTPN22 (Ellis et al., 2014), VTCN1, TNFA and MIF are also associated with JIA (Prahalad and Glass, 2008) (Reinards et al., 2014) .

Environmental factors

Infectious agents seem to be the most important environmental factor to trigger JIA. There is evidence that infections with streptococcus trigger the onset of disease (Barash and Goldzweig, 2007). Joint inflammation is usually observed two weeks after streptococcus infection. It is based on a cross-reactive immune response between a self protein and a protein of exogenous origin, in this case of bacterial origin (known as molecular mimicry). Other triggering factors discussed are viral

infections with Parvovirus B19 and Epstein-Bar-Virus, lack of vitamin D, stress or vaccination (Ellis et al., 2010)

JIA is an autoimmune disease

Joint inflammation in JIA is due to a failure of the immune system. Under physiological conditions, the immune system recognizes its own constituents as “self” and induces an immune response to everything that is “non-self”. T lymphocytes that react to autoantigens are usually eliminated during the maturation in the thymus. However, some autoreactive T cells escape thymic censorship and are released into the peripheral blood. In healthy conditons additional mechanims in the periphery, such as anergy or regulatory T cells, silence these autoreactive T cells. In autoimmune disease either the thymus fails to eliminate autoreactive T cells, or peripheral tolerance is unsuccessful. Autoreactive T cells can also derive from T cells that mistakenly recognize translationally modified autoantigens as “non-self”. Depending on the type of antigen, autoreactive T cell induce inflammation in various target organs. In the case of JIA, autoreactive T cells reach the joint and recognize self-antigens presented by resident antigen presenting cells. Antigens are recognized by T lymphocytes with a specific receptor (TCR). Through receptor binding of the antigen, an intracellular cascade of signalling molecules is induced, leading to a variety of T cell effector functions. There are indications that JIA is a T cell- driven autoimmune disease: First, activated and oligoclonal expanded T cells accumulate in the synovial fluid of patients with acute JIA (Thompson et al., 1998) (Wedderburn et al., 2001). Second, JIA is associated with certain HLA alleles. Third, in some subtypes of JIA the severity of disease correlates with the frequency of effector T cell subsets in the synovial fluid (Nistala et al., 2008).

Differentiation of CD4+ effector T cells

T cells originate from the bone marrow and mature in the thymus. Mature T cells enter the bloodstream and migrate to lymphoid organs. There, naïve CD4+ T cells recognize antigenic peptides presented by HLA class II molecule on the surface of an antigen-presenting cell (APC). Upon antigen recognition, CD4+ T cells are induced to proliferate and differentiate into effector T cells and long-lived memory cells. Depending on the cytokine milieu that is mainly provided by the priming APC, mature

naïve CD4+ T lymphocytes develop into functionally distinct effector cell subsets, namely T-helper (Th) cells, for example Th1, Th2 or Th17 cells, or regulatory T cells (Tregs) (Figure 1).

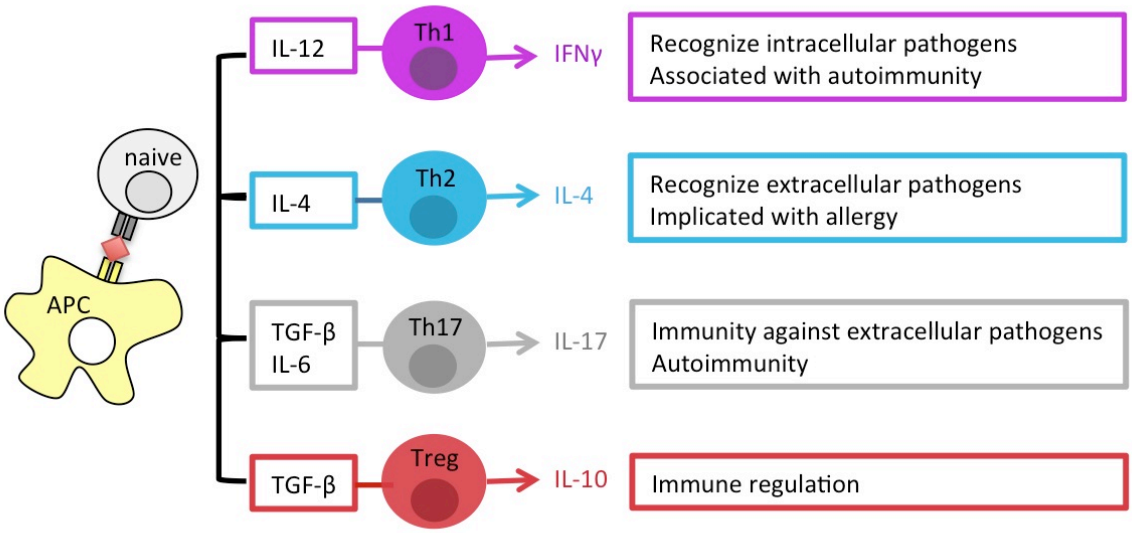

Figure 1 Differentiation of naive CD4+ T cells into different effector lineages.
After encountering with a specific antigen presented by an APC, mature naive T cells develop into different T cell lines depending on the cytokine milieu produced by the APC. Effector T cells are distinguished according to the cytokines they produce and to their distinct functions in the adaptive immune response.

In the case of Th1 development, the cytokine interleukin (IL)-12 is the priming factor in the local environment. Th1 cells are responsible for cell-mediated immunity and recognize intracellular pathogens. Th2 cells develop in the presence of IL-4 and are essential for the clearance of extracellular pathogens. However, both cell subsets have also been associated with pathological responses, Th1 cells with autoimmunity and Th2 cells with allergy (Murphy, 2008). The most recently identified CD4+ effector cells are Th17 cells, characterised by the ability to produce IL-17, and to a lesser extent IL-22. The polarizing factor for Th17 development is IL-6 in the presence of transforming growth factor β (TGFβ) and IL-23. Th17 cells always express CD161 and the chemokine receptor (CCR) 6, although these molecules are not exclusive for this cell type. A broad range of cells including fibroblasts, epithelial cells and keratinocytes express the IL-17 receptor. Through interaction of IL-17 with its receptor on stromal cells, further inflammatory chemokines and cytokines are produced resulting in the recruitment of neutrophils and monocytes to the site of

inflammation. Thus, Th17 cells amplify the acute inflammatory response by activating parts of the innate immune system. Concerning effector function, Th17 cells have been associated to mediate immunity against extracellular pathogens (Peck and Mellins, 2010). Increased amounts of Th17 cells have also been found in many autoimmune diseases including inflammatory bowel disease, MS and arthritis (Annunziato et al., 2008) (Brucklacher-Waldert 2009) (Awasthi and Kuchroo, 2009).

Regulatory T cells are essential for T cell tolerance against self-antigens and for the regulation of exacerbated immune responses. Impaired Treg function leads to the development of autoimmune diseases in mice and human. Tregs control exacerbated immune responses by the secretion of inhibitory cytokines, such as IL-10 and TGFβ, and by the removal of growing factors, which are required for effector T cell activity (reviewed by (Vignali et al., 2008). Tregs may originate in the thymus or can be induced in the periphery, provided that naïve cells are activated in the presence of TGFβ but no additional inflammatory cytokines. They are identified by the expression of the transcription factor Forkhead Box Protein 3. Human Tregs can be phenotypically identified by the surface expression of CD4, high levels of CD25 and the absence of CD127 (Miyara et al., 2011).

For a long time it has been postulated that T cell development into different effector subset is unidirectional and lineage committed. Only recently a certain degree of plasticity between the CD4+ T cell lineages has been observed, indicating that a T cell that has been tuned to produce a certain cytokine can be promped in another direction of immune reponse (reviewed by (O'Shea and Paul, 2010). The ability to direct T cell differentiation possibly facilitates new target options in T cell mediated diseases such as JIA.

Activation and maturation of T cells

After TCR engagement with an APC, T cell express activation markers, including chemokine and cytokine receptors, adhesion molecules and co-stimulatory molecules. While CD69 and CD25 are upregulated quickly after stimualtion, CD38 and CD26 are markers that are expressed on T cells that have been stimulated for days. CD69 is a transient marker, while CD25 stays upregulated for a longer time period (Figure 2).

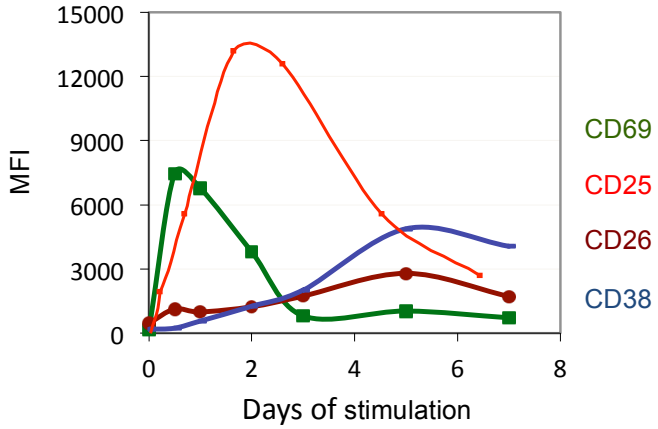

Figure 2 Activation markers expressed on T cells after stimulation.
Shown in the figure is the upregualtion of different activation markers in relation to the days of in vitro stimualtion of T cells. While CD69 and CD25 are early upregulated after stimualtion, CD26 and CD38 are expressed after various days of stimualtion. Adapted from (Meyer Tolosa, unpublished).

The number of effector T cells peaks about one week after antigen recognition. Afterwards the majority of antigen specific T cells dies and less than 10% develop into memory cells. Memory T cell are distinguished into central memory (CM) and two effector memory (EM) subtypes. Central memory T cells express CD45RO, CD62L, CCR7 and CD27 allowing them to migrate into lymph nodes and lymphoid tissue. They rapidly proliferate and differentiate into effector T cells following antigen stimulation, but lack immediate ability to produce inflammatory cytokines. Effector memory cells are CD45RO+, CD62L-, CCR7-, CD27 +/- and express homing receptors that facilitate migration to non-lymphoid sites of inflammaton. In contrast to central memory cells they are cytotoxic and capable of producing interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), IL-4 and IL-5 upon stimulation. Terminally differentiated effector memory cells (TEMRA) are CD45RA+ and resistent to stimulation. In the condition of JIA, the frequencies of T cells in the naive, CM, EM and TEMRA compartment may reflect the inflammatory state in these children.

Cellular pathogenesis of different forms of JIA

The pathogenesis of the distinct JIA subtypes are likely very different. Especially, oligo- and polyarthritis differ from systemic JIA, in the sense that children with systemic JIA struggle with inflammatory processes in multiple compartments whereas in patients with other entities the inflammation is basically restricted to the joint (Figure 3).

Systemic JIA

Systemic JIA is characterised by an uncontrolled activation of the innate immune system, resulting in granulocytosis, thrombocytosis and an increase of acute phase proteins in the peripheral blood. In contrast to poly- and oligoarthritis, no autoreactive T cells can be found and so far no association with HLA genes has been described.

Through engagement with danger molecules, monocytes, macrophages and neutrophils start to produce high levels of inflammatory cytokines such as IL-6, IL-18 and IL-1, leading to the clinical symptoms of fever, skin rash, hepatosplenomegaly and serositis (Pascual et al., 2005, de Jager et al., 2007). Proinflammatory proteins like S100-A8, -A9 and -A12 are extensively increased in the blood. The augmentation of these S100 proteins is unique to the systemic form of JIA, since only slight changes in S100 proteins can be found in all other subtypes.

IL-1, IL-18 and the S100 proteins are released over an alternative secretory pathway, which differs from the classical intracellular transport mechanism via the endoplasmatic reticulum and Golgi complex used by other cytokines: Proteins are translated into precursor forms within the cytosol. In a second step, that includes the activation of the P2X7 receptor by adenosine triphosphate (ATP) and other mediators of sterile inflammation, caspases of the inflammasome convert these proteins into the active forms (Frosch and Roth, 2008). Loss of control in these mechanisms may be associated with the systemic inflammation in JIA. Taken together, systemic JIA shows more features of a non-T cell mediated, rather autoinflammatory disease than of a classical antigen-driven autoimmune disease.

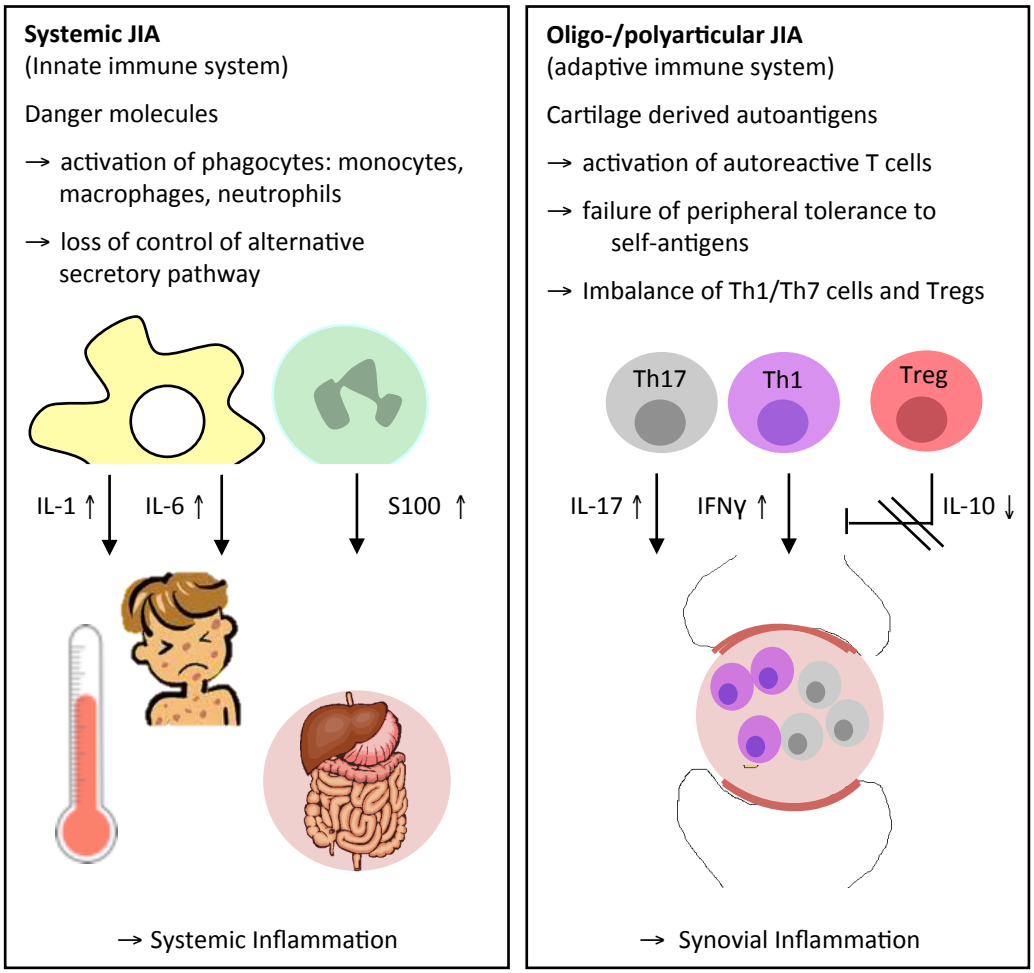

Figure 3 Pathogenesis of oligo- and polyarthritis vs. systemic JIA.
In poly- and oligoarthritis the activation of autoreactive T cells and the failure of regulatory T cells lead to synovial inflammation. In contrast, the systemic form of JIA contains elements of a chronic inflammatory disease showing signs of systemic inflammation. In this subtype it is the innate immune system that plays a more prominent role. Adapted from (Lin et al., 2011)

Pathogenesis of poly- and oligoarthritis

Poly- and oligoarthritis are classical antigen-driven and lymphocyte-mediated autoimmune entities. Cartilage-derived autoantigens such as aggrecan, fibrillin and matrix metalloprotease-3 could function as CD4+ T cell activators (Kamphuis et al., 2006). Characteristic of both entities is the high amount of autoreactive T cells in the synovial fluids of patients. Autoantibodies such as rheumatoid factor and antinuclear antibodies (ANAs) are found in both disease subtypes. While the presence of rheumatoid factor is usually only used for diagnostic purposes, the presence of ANAs is linked to the course of disease (Ravelli et al., 2011).

As in other autoimmune diseases, Th1 and Th17 cells are the pathogenic cells leading to increased amounts of IFNγ and IL-17 in the synovial fluid. Increased amounts of IL-17 have been found in the serum of JIA patients with active disease

compared to the ones in remission (de Jager et al., 2007). Also patients with more severe subtypes such as extended oligoarthritis show higher amounts of IL-17 in their inflamed joints (Nistala et al., 2008). IL-17 acts on synovial fibroblasts to stimulate the release of matrix-metallo-proteinases, which directly destroy cartilage. Together with IL-1, IL-17 induces osteoclast differentiation, which consequently leads to bone erosions. Additionally, IL-17, IL-1 and TNFα stimulate the cytokine production of monocytes (Lin et al., 2011). Importantly, once inflammation in the joint is initiated, IL-17 can maintain the disease independently from other proinflammatory cytokines (Koenders et al., 2006).

However, it is now known that the phenotype of Th17 cells is not stable, and Th17 cells that were isolated from children with active JIA, could be induced to change into a phenotype that combines Th17 and Th1 (Th17/Th1) cell features *in vitro,* if cultured in conditions that mimic the disease site (low concentration of TGFβ and high concentration of IL-12) (Nistala et al., 2010). Of note most IL-17-producing T cells in the synovial fluid of patients of JIA show a Th17/Th1 phenotype and only few IL17+ IFNγ- T cells can be found in inflamed joints (Cosmi et al., 2011). These findings are particularly notable concerning therapeutic options in which the ability to change IL-17-producing cells into a less aggressive phenotype would be a promising approach. Additionally the study of other cell subsets that can produce IL-17, namely TCRγδ+ T cells and recently identified mucosal-associated invariant T (MAIT) cells are an interesting study targets because they may contribute to the pathogenesis of poly- and oligoarthritis.

Imbalance of regulatory and inflammatory effector cells in poly- and oligoarthritis

In healthy conditions, regulatory cell subsets ensure that the immune system is not over activated. In JIA, the impaired function of regulatory cells lead to an excess of T cell activity in the joints, resulting in inflammation. The imbalanced frequency and function of regulatory and inflammatory cells is interesting in the context of new therapy options that could target to restore a more favourable state. Figure 4 shows a schema of how leukocyte subsets can be grouped according to their phenotype, effector function and contribution to the imbalance of regulation and inflammation seen in the joints of JIA patients. The role of innate-like-lymphocytes, namely

TCRγδ+ T cells and MAIT cells, is still unclear, challenging the classification into one or the other group. Also the function of ectoenzyme expressing T cells in inflammation is not fully understood, so that the categorization is difficult.

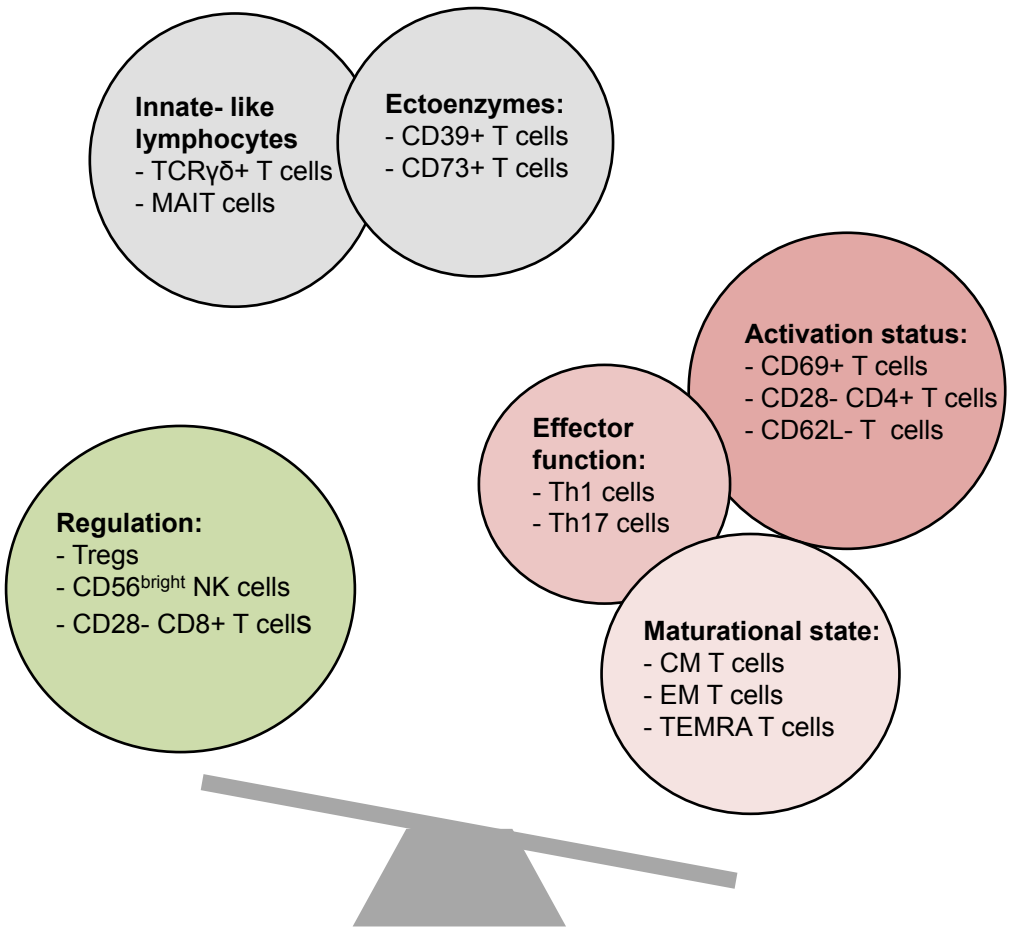

Figure 4 Overview of leukocyte subsets with regulatory, inflammatory or unknown function. An imbalanced frequency or impaired function of regulatory and inflammatory subsets may lead to the inflammatory processes observed in the joints of patients with poly- or oligoarthritis JIA subtypes. Lymphocyte subsets in the red circle mark populations that are associated with inflammation while cell subsets in the green circle have immune regulatory functions. Cell subsets in the grey circle cannot clearly be classified to one or the other group yet.

Regulatory T cells in the joints of JIA patients

The data on the frequency and function of Tregs in JIA are still controversial. Conflicting data about the frequency of Tregs in peripheral blood of JIA patients have been reported. De Kleer and colleagues have shown that children with oligoarthritis have a reduced number of regulatory T cells in the blood compared to healthy donors (de Kleer et al., 2004), whereas recent studies describe higher percentages of Tregs in the peripheral blood of JIA patients with active disease (Wu et al., 2014).

However, in the synovial fluid there is consisting data about an increased frequency of Tregs (de Kleer, Wedderburn et al. 2004) (Nistala et al., 2008).

Recently, the presence or absence of the ectonuclease CD39 is used to distinguish two subsets of Tregs. Interestingly, CD39+ Tregs are superior at suppressing IL-17 production by T cells compared to CD39- Treg (Fletcher et al., 2009), and thus they are likely to play a role in the control of the autoimmune response in diseases such as MS and JIA. Decreased amounts of CD39+ Tregs have been found in the peripheral blood of patients with MS (Borsellino et al., 2007), while in JIA CD39+ Tregs are increased in the syovial fluid (Moncrieffe et al., 2010). Why inflammation cannot be controlled despite an accumulation of Tregs in the synovial fluid is unclear. A different study argues against an insufficient suppressive function of Tregs as the source of synovial inflammation and rather points out the influences of an inflammatory milieu leading to a decreased susceptibility of effector T cells in target organs (Haufe et al., 2011).

In summary, uncontrolled inflammation in the joints of JIA patients is probably due to an insufficient frequency of potent Tregs and the hyporesponsiveness of effector T cells within inflamed joints of JIA patients.

Influences of ectoenzymes to harmonize inflammatory processes

CD39 is an ectoenzyme that hydrolyses ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Figure 5) and is expressed on T lymphocytes. Because of the ability to eliminate proinflammatory extracellular ATP, CD39 is an interesting molecule in the context of inflammation. In the synovial fluid of JIA patients, the frequency of conventional CD39 expressing CD4+ and CD8+ lymphocytes is increased and associated with higher ATPase activity (Moncrieffe et al., 2010).

CD73, an ectonuclease with a specific AMPase activity, metabolizes extracellular AMP into adenosine and through this generates an anti-inflammatory molecule. Adenosine binds to the Adenosine 2A (A2A) receptor on T cells and inhibits the production of IL-2 and IFNγ through intracellular signalling. Consequently the generation and proliferation of effector T cells is restricted. In mice it has been shown that adenosine also has an influence on T cell differentiation. Adenosine inhibits the generation of IL-17-producing cells in reducing IL-6 expression and in up regulating TGFβ (Zarek et al., 2008). Since high amounts of TGFβ are a stimulus for

naïve cells to develop into Tregs, adenosine thus promotes T cell differentiation away from Th17 cell and towards the induction of regulatory cells. Synovial cells of JIA patients show low expression levels of CD73 (Moncrieffe et al., 2010) (Botta Gordon-Smith et al., 2014).

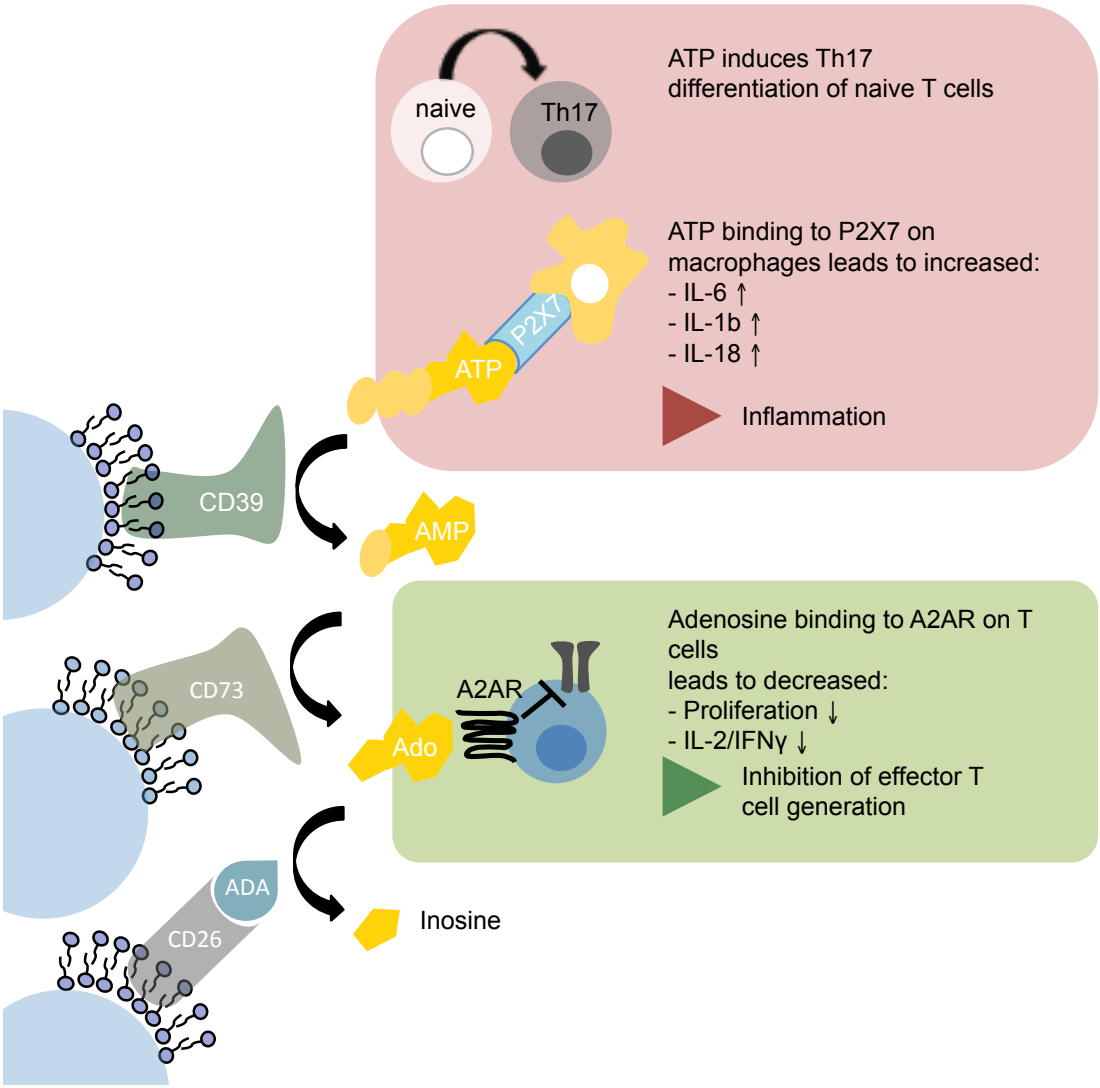

Figure 5 Scheme of the interaction between the ectoenzymes CD39, CD73 and CD26.
The subsequential action of CD39 and CD73 cleaves ATP, which is associated with several proinflammatory functions, into adenosine. Through binding to the A2A receptor adenosine induces several anti- inflammatory effects such as a decrease in T cell proliferation or the induction of anti-inflammatory effector T cells. ADA, which is bound to CD26, can eliminate this anti-inflammatory effect by metabolizing adenosine into inosine. Adapted from (Zhang, 2010)

The adenosine-deaminase (ADA) can further metabolize adenosine into inosine and take away the anti-inflammatory signal. Morrison et al. have found a binding site of ADA on the surface molecule CD26 although it is unclear if the ectoenzyme is always

bound to ADA. CD26 is an ectoenzyme that cleaves proline dipeptides. It serves as an adhesion receptor on epithelial cells, facilitating migration through the endothelium. In humans, $CD26^{high}$ hematopoietic cells seem to play a role during inflammation since they secrete IFNγ and IL-2 (De Meester et al., 1999). The interaction of these ectoenzymes and the question how this is regulated is especially interesting in autoimmune disease. Since in JIA we have direct access to the inflammatory site, it is an ideal disease to study ectoenzymes.

Immunosenescene in patients with JIA

Chronic inflammation in the joints of patients with JIA can also be reflected by an unusual composition of naive and memory T cells compared to healthy children of same age. Premature aging of the immune system, also know as immunosenescence, has been described in adult autoimmune diseases such as MS and rheumatoid arthritis.

Prelog and collegues have studied the frequency of naive T cells in JIA in relation to age. In the peripheral blood of JIA patients they describe a reduced frequency of naive CD45RA+ CD62L+ T cells and a compensatory increase in CD45RO+ memory T cells compared to healthy children of same age (Prelog et al., 2008). There is only one group that has studied the frequency of memory T cells in the synovial fluid. Gattorno and collegues describe an enrichment of CD4+ CD45RO+ CD27- effector memory T cells in the joints of JIA patients compared to peripheral blood lymphocytes (Gattorno et al., 2002) (Gattorno et al., 2005).

Taken together it is clear that in JIA there is an earlier shift to memory T cells. Although a specific antigen has not been identified, T cells are chronically stimulated leading to an accumulation of effector memory T cells at the site of inflammation and a premature aging of the immune system in children with JIA.

Immunosenescene can also be reflected by an increase of repeatedly stimulated cells that are characterised by the loss of CD28. CD28 is a co-stimualtory molecule that receives signals from its ligand CD80 and CD86 expressed on APCs. If T cells do not receive this second signal they become anergic. In healthy conditions the frequency of CD4+ CD28- T cells increases physiologically with age (Miyazaki et al., 2008). Interestingly a greater expanision of CD4+ T cells that lack CD28 has been described in the blood of patients in multiple autoimmune diseases such as

rheumatoid arthritis, MS (Miyazaki et al., 2008), Wegner's granulomatosis (Moosig et al., 1998) and inflammatory bowel disease (Probert et al., 1996) .

Isolated CD4+ CD28- T cells from patients with arthritis are not anergic but produce large amounts of IFNγ, TNFα and IL-2 upon only little *in vitro* stimulation (Fasth et al., 2004). Phenotypically they are characterised by the expression of memory surface markers (CD45RO) and by the lack of activation markers (CD69-). CD4+ CD28- T cells are mainly found in the blood of patients with rheumatoid arthritis and only in some cases can be found in the joint. Thus, migration of these potent effecor cells to their target organs is possible but seems to be highly regulated (Fasth et al., 2004).

The influence of the innate immune system to regulatory processes in the joints

Natural Killer (NK) cells are lymphocytes that belong to the innate immune system and have a number of effector functions that are important for an early host defense against bacterial and viral pathogens: they recognize and lyse virus- infected and tumor cells and produce proinflammatory cytokines.

Phenotypically they are characterised by the presence of CD56 and the lack of CD3. Based on the expression of CD56 two subsets of NK cells have been identified: $CD56^{bright}$ NK cells are $CD56^{high}$ and CD16- whereas and $CD56^{dim}$ NK cells express low levels of CD56 and high amounts of CD16. In human peripheral blood more than 90% are $CD56^{dim}$ whereas $CD56^{bright}$ cells are the major subsets in lymph nodes. Apart from their phenotype they also differ functionally. $CD56^{bright}$ NK cells produce large amouts of cytokines such as IFNγ, TNFβ, IL-10 and Granulocyte macrophage colony-stimulating factor (Cooper et al., 2001) whereas $CD56^{dim}$ NK cells are potent cytotoxic effector cells expressing high levels of perforin and granzyme. An accumulation of $CD56^{bright}$ NK cells have been found in the target organs of autoimmune diseases such as MS and rheumatoid arthritis (Dalbeth and Callan, 2002).

A dysfunction of NK cells has been described in patients with systemic JIA. Compared to all other subtypes of JIA, patients with sJIA have a reduced frequency of NK cells and an impaired cytotoxic NK cell function characterised by reduced perforin and granzyme B expression (Zhou et al., 2013). Depressed NK cell activity is

likely to be relevant to the pathogenesis of the macrophage activation syndrome in sJIA (Villanueva et al., 2005).

The role of innate-like lymphocytes in synovial inflammation

Innate-like lymphocytes represent a group of cell types that share characteristics of both the innate and adaptive immune system. They express receptors with limited antigen diversity but can rapidly respond to exogenous pathogens without long lasting activation processes including clonal expansion. Invariant natural- killer T cells (NKT), TCRγδ+ T cells and MAIT cells belong to this unique cell subset acting as "first-line" immune response and representing a bridge between innate and adaptive immune system. Their role in health and disease is not fully understood but there is evidence that these cells play an important role in autoimmune diseases, partly due to their potentital to produce IL-17 and other profinflammatory cytokines.

TCRγδ+ T cells

The TCR of conventional CD4+ or CD8+ T cells consists of an α- and a β- chain, named TCRαβ+ T cells. A minority of T cells uses a γ- and a δ- chain instead, and are thus named TCRγδ+ T cells. These cells also derive from the thymus, but little is known about their intrathymic development and selection processes. In the mouse, they leave the thymus in several waves to colonize mucosal interphases, mostly before conventional TCRαβ+ T cells undergo selection (Moens et al., 2011). Many questions still remain unclear about their function, activation and regulation, especially in young children.

In healthy humans TCRγδ+ T cells constitute 2-10% of T cells in peripheral blood. There are at least two subtypes, the Vδ1 and Vδ2 population. In adults, the Vδ2 chain preferentially pairs with the Vγ9 chain, whereas the Vδ1 chain assembles with any other γ chain. The Vδ2 subtype is mainly found in the blood, while Vδ1 T cells are more abundant in epithelial sites such as the mucosa or the skin, where they contribute to the first line immune defence against pathogens. Human Vδ2 T cells respond rapidly to Mycobacteria-derived phosphoantigens with proliferation. In contrast, Vδ1 T cells are activated by the ligands MICA and MICB on neighbouring epithelial cells (Berkun et al., 2011) (Moens et al., 2011). In mice, Toll-like receptor engagement and IL-23 can trigger activation of TCRγδ+ T cells (Martin B, Immunity 2010), suggesting that these cells can provide a much faster response than the

classical TCRαβ+ T cells. Upon activation, TCRγδ+ T cells produce IFNγ and cytotoxic mediators such as perforin and granzymes. Moreover, TCRγδ+ T cells seem to play a role in the immune defence of very young children, since already at birth a majority of TCRγδ+ T cells do not show a naive phenotype and, in contrast to conventional TCRαβ+ T cells, are able to produce IFNγ immediately upon stimulation (De Rosa et al., 2004).

In addition to IFNγ, murine TCRγδ+ T cells have been shown to produce IL-17. Until now there are only few publications addressing IL-17 production by human TCRγδ+ T cells: Moens el al. describe that neonatal TCRγδ+ T cells can be polarized to produce almost exclusively IL-17 when cultured with IL-23 and zoledronate, an aminobiophosphonate that constitutes a stimulus for neonatal Vδ2 T cells. In contrast, TCRγδ+ T cells from adult blood in the same conditions did not produce any IL-17, again suggesting that IL-17-producing TCRγδ+ T cells could play a role in the defence of early life infections, compensating for the not fully developed adaptive immune system (Moens et al., 2011).

In parallel, the group of Caccamo used IL-1b, IL-6 and TGFβ to polarize human naive TCRγδ+ T cells to produce exclusively IL-17. They also showed that in children with bacterial meningitis the amount of IL-17-producing Vγ9δ2 T cells was significantly enriched when compared to healthy donors. Of note, in the cerebrospinal fluid of those children more than 70% of the TCRγδ+ T cells were IL-17-producing Vγ9δ2 (Caccamo et al., 2011).

Patients with active ankolysing spondylitis show a striking increase of IL-17-producing TCRγδ+ T cells in peripheral blood compared to healthy donors or patients with rheumatoid arthritis. Interestingly only IL23R+ TCRγδ+ T cells secrete IL-17 and almost all of these cells co- express CD161 and CCR6 (Kenna et al., 2012).

Conflicting data has been published concerning the function of TCRγδ+ T cells in JIA. Recently it has been shown that TCRγδ+ T cells of both subtypes produce large amounts of TNFα and IFNγ in the peripheral blood and the synovial fluid of JIA patients. Similar as in CD4+ T cells only a small proportion of Vδ1 and Vδ2 cells in the synovial fluid produced IL-17. The same was observed in peripheral blood with the exception of Vδ1 cells, where IL-17 production was observed in 30% of all cells (Bendersky et al., 2012). In summary this data suggests that TCRγδ+T cell can greatly contribute to the proinflammatory cytokine milieu in both peripheral blood and synovial fluid.

In contrast to that, Berkun and colleagues suggest a rather regulatory function of TCRγδ+ T cells in JIA. They reported that high frequencies of synovial fluid Vδ1 and Vδ2 T cells correlate with late onset of disease, longer remission periods and the use of milder medication and thus have protective effects to disease progression (Berkun et al., 2011). In the same line, Chomarat et al. found that TCRγδ+ T cells showed increased levels of IL-4 compared to TCRαβ+ T cells in the synovial fluid and the peripheral blood of arthritis patients, pointing to a more regulatory role of TCRγδ+ T cells (Chomarat et al., 1994).

MAIT cells

Human MAIT cells express the invariant T cell receptor chain Vα7.2 preferentially combined with a limited number of β- chains (Vβ2 and Vβ13). The majority of MAIT cells are CD8+ positive, only about 10% are double negative and very few MAIT cells express CD4 (Walker et al., 2012). In human blood they represent 1-8% of all T lymphocytes (Martin et al., 2009b) whereas in the liver 20-45% of T cells belong to the MAIT subtype (Dusseaux et al., 2011). MAIT cells found in cord blood have a naive phenotype whereas most MAIT cells found in adults express a memory status (CD45RO+ $CCR7^{low}$, $CD62L^{low}$) (Dusseaux et al., 2011). Unlike conventional T cells, MAIT cells are activated independedly of the classical MHC class I and class II antigen-presentation pathway. Bacteria and yeast infected antigen presenting cells, which are situated in the periphery, activate MAIT cell through antigen presentation on the highly phylogenetically conserved major histocompatibility complex (MHC) class I–related molecule MR1. The intrathymic selection process of MAIT cells is not fully understood, although it seems like MR1 expressing non-B and non-T cells are involved in the selection steps: these could be dendritic cells or macrophages (Gold et al., 2010). Similar to TCRγδ+ T cells, MAIT cells recognize antigens of the vitamin B pathways of bacteria and yeast (Kjer-Nielsen et al., 2012). Upon stimualtion they produce IFNγ, TNFα and IL-17. Interestingly they share differentiation factors of Th17 cells inlcuding cytokine production (IL-17 and IL-22), transcription factor (RORγt), and the expression of CCR6, IL23R and CD161 on the surface. Furthermore, they highly express CD26.

MAIT cell have been found in the target organs in a variety of diseases. An accumulation of MAITs cells has been described in lesions of the central nervous system from MS autopsy samples, as well as in human kindney and brain tumors.

Patients with pulmonary bacterial diseases have a decreased frequeny of MAIT cells in peripheral blood whereas an accumulation has been found in pulmonary lesions representing the site of inflammation (Le Bourhis et al., 2010). These resulIts indicate that MAIT cells may migrate to the site of inflammation and function with antimicrobacterial properties.

Patients with MS have a reduced frequency of MAIT cells in peripheral blood compared to healthy donors, especially in times of disease relapses. The expression of CCR5, CCR6 and α4β1 integrin may facilitate infiltration into the brain. The function of MAIT cells in MS is unclear. In contrast to their ability to produce proinflammatory cytokines it has also been shown that they are able to suppress IFNγ-producing T cells in an IL-10 and TGF-β independend way (Miyazaki et al., 2011).

Concerning arthritis, MAIT cells have only been described in the animal model of collagen-induced arthritis. Chiba et al. have shown that mice lacking the MR1 molecule that consequently do not have MAIT cells show a decreased severity of CIA. Adoptive transfer of MAIT cell augmentes arthritis in the same animals, indicating that MAIT cell may contribute to the progression of disease (Chiba et al., 2012). In rheumatoid arthritis and JIA, MAIT cell have not been studied yet.

Therapy

Treatment options for JIA include a variety of classical anti-inflammatory drugs and an increasing number of biologic agents, also called biologics (reviewed by (Haines, 2007). Nonsteroidal anti-inflammatory drugs (NSAIDs) have been the most popular treatment of JIA for decades. NSAIDs block cyclooxygenases preventing the formation of prostaglandins and show effects in pain reduction and reduced inflammation in the joints. Although being the first line of treatment, the efficiency of NSAIDs is not very high and side effects as gastritis have to be taken in consideration. If NSAID treatment fails, intra-articular injections of corticosteroids are a good option to continue treatment. They are safe and well tolerated and show improvement of synovitis without any structural damage to the joint. However, since children are usually anesthetized during the procedure, the risk that comes with any anaesthesia has to be considered.

Methotrexate has found its place as a second line treatment option due to its proven effectiveness. It functions as an analogue of folic acid and belongs to the group of cytostatic drugs. The main mechanism of methotrxate in the treatment of arthritis seems to be the inhibition of enzymes involved in the purine metabolism leading to a consequent accumulation of immune regulatory adenosine. Other so-called disease modifying drugs (DMARDs) are Azathioprine (a purine analogue) and Sulfasalazine (a pyridin), which is also used in the treatment of inflammatory bowl disease.

The most recently developed group of pharmaceutics are biologic agents, which target specific cytokines or other molecules playing a role in the pathogenesis of JIA, mostly TNFα. TNFα is one of the major proinflammatory cytokines in patients with oligo- and polyarthritis. Therefore medicaments that result in a decrease of TNFα signalling were a promising therapy option. In the treatment of adult rheumatoid arthritis they have shown impressive results. A variety of anti-TNFα treatments are available today: Etanercept is a chimeric molecule of a soluble TNFα receptor joined with an Fc fragment of IgG1. It is injected subcutaneously once a week and is the only anti-TNFα therapy that is approved for children. Infliximab and Adalimumab are both monoclonal antibodies against TNFα with the difference that Adalimumab is a fully humanized antibody, whereas Infliximab is a mouse/human chimera. Infliximab and Adalimumab are injected intravenously and subcutaneously, respectively. Both medications are only used off label in paediatric patients and require additional surveillance of laboratory parameters such as liver enzymes.

In systemic JIA the use of TNFα blockage is not successful, indicating that this molecule does not have such a big impact on the pathogenesis compared to the other subtypes of JIA (Quartier et al., 2003). Better therapeutic results in systemic JIA are achieved with the blockade of the IL-1 receptor (IL-1R) and with IL-6 receptor (IL-6R) antagonists, implicating that these cytokines are more relevant in systemic JIA. Anakinra is a recombinant IL-1R antagonist that inhibits the production of prostaglandins and proinflammatory cytokines. However, Anakinra has shown poor effects treating children with JIA subtypes other than systemic JIA. Tocilizumab is a humanized monoclonal anti- IL-6R antibody and, like Anakinra, shows very good results in treating patients only with systemic JIA, the most challenging subtype to treat so far (Herlin, 2010).

The most recent biologic agent introduced for the treatment of rheumatoid arthritis is Abatacept, a fusion protein containing the extracellular part of human CTLA-4 and the Fc part of IgG-1 that interferes with T cell activation. CTLA-4, giving an inhibitory signal, binds with higher affinity to CD80 and CD86 on APCs. Thus, T cells cannot bind to the APC and T cell activation is disrupted. Abatacept has been successfully tried in the treatment of polyarticular JIA. Since it is a rather new medication, long-term side effects are still unknown and patients treated with Abatacept have to be followed up closely (Goldzweig and Hashkes, 2011).

AIM OF THE STUDY

Biologic agents are only used in patients that have been unsucessfully treated with NSAIDs and DMARDs. Why some patients need a more aggressive therapeutic approach, while others can be treated with milder medication, still remains unclear. Current knowledge about the pathogenesis and progression of disease does not allow us to identify JIA patients that will need treatment with biologic agents and differentiate them from others. The fact that JIA is a very heterogenous disease and that the classification of subgroups has been changed numerous time, does even complicate the issue.

Therefore it would be ideal to find a combination of markers that would indicate the severity of disease progression or predict the indication of an early introduction of biologic agents. So far there have been no studies integrating a variety of immune cell types in the peripheral blood and synovial fluid of JIA patients, but immunological studies in JIA have only focused on the analysis of single cell types. In this study we want to phenotype JIA patients in detail including a variety of cell subsets and markers for inflammation, effector function, regulation and maturation. From this we hope to get insight into the interaction of regulatory and inflammatory leukocytes, to contribute to the knowledge of pathogenesis in JIA and to identify new potential targets of therapy. For this, we first had to write a study protocol and summit it to the local health authorities for approval. With the samples collected, we wanted to

1. Establish a FACS-staining protocol to identify inflammatory and regulatory myeloid and lymphoid cells and to evaluate the activation status, cytokine production and maturational status using flow cytometry,
2. Perform a detailed characterisation of frequencies and phenotype of regulatory and effector leukocytes in peripheral blood of healthy donors and different disease entities,
3. Compare frequency and phenotype in the blood and the synovial fluid from patients that are punctured,
4. Analyse these cell types in the context of different treatments received by the patients.

MATERIAL AND METHODS

Material

Antibodies

Fluorochrome labeled antigen	*Clone*	*Company*	*Catalog number*
CD3- V450	UCHT1	eBioscience	#480038
CD3- FITC	UCHT1	BD	#557694
CD3- PECy5.5	OKT3	eBioscience	#45003742
CD4- APCCy7	RPA-T4	Biolegend	#300518
CD4- V510	RPA-T4	BD	#560768
CD4- Pacific Blue	RPA-T4	Biolegend	#300521
CD4- FITC	RPA-T4	Biolegend	#300519
CD4- APC	SK3	Biolegend	#344613
CD8- Pacific Blue	RPA-T8	Biolegend	#301033
CD8- V510	RPA-T8	BD	#560774
CD8- Brilliant Violett 421	RPA-T8	Biolegend	#301035
CD11c- APC	3.9.	eBioscience	#17011673
CD14- V450	M5E2	BD	#560349
CD14- Pacific Blue	HCD14	Biolegend	#325616
CD14- FITC	M5E2	BD	#555397
CD16- FITC	3G8	Biolegend	#302006
CD16- APC Cy7	3G8	Biolegend	#302018
CD19- FITC	HIB19	BD	#555412
CD19- PE Cy 7	HIB19	BD	#560728
CD19- PE Cy 7	HIB19	Biolegend	#302216
CD20- V450	L27	BD	#642274
CD25- PE	2A3	BD	#555432
CD25- Brilliant Violet 421	BC96	Biolegend	#302629
CD26- FITC	M-A261	BD	#555436
CD27- APC Cy7	O323	Biolegend	#302816
CD27- APC H7	M-T271	BD	#560222
CD28- PECy7	CD28.2	Biolegend	#302926
CD38- FITC	HIT2	BD	#555459
CD39- PE Cy 7	A1	Biolegend	#328212
CD40L- FITC	24-31	eBioscience	#111548
CD45RA -APC	HI100	eBioscience	#170458
CD45- V510	HI30	BD	#560777
CD56- PE Cy 7	B159	BD	#557747
CD56- APC	N901	Beckman Coulter	#IM2474
CD57- FITC	NK-1	BD	#555619
CD62L- PE	DREG-56	BD	#555544
CD69- APCCy7	FN50	Biolegend	#310914
CD69- APC Cy7	FN50	BD	#557756
CD69- PE Cy 7	FN50	eBioscience	#25-0699
CD69- PeCy7	FN50	Biolegend	#310914
CD69-APC	FN50	BD	#560967
CD73- PE	AD2	Biolegend	#344004
CD80- PE	L307.4	BD	# 557227
CD86- APC	2331	BD	# 555660
CD123- PECy7	6H6	Biolegend	#306010
CD127- PE Cy5.5	HCD127	Biolegend	#317610

Fluorochrome labeled antigen	*Clone*	*Company*	*Catalog number*
CD161- PE	HP-3G10	eBioscience	# 12161942
CD161- APC	HP-3G10	eBioscience	#339910
CCR4- PE Cy 7	TG6	Biolegend	#335405
CCR6- CD196 PE	R6H1	eBioscience	#121969
CCR6- PERCy5.5	Tg7/CCR6	Biolegend	#335505
CCR7- APC	3D12	BD	557734
CXCR3- FITC	1C6/CXCR3	BD	#558047
γδ-TCR- FITC	11F2	BD	#347903
γδ-TCR- PE	11F2	BD	#333141
Vδ2- FITC	IMMU 389	Beckman Coulter	#IM1464
HLA-DR- Pacific Blue	L243	Biolegend	#307624
HLA-DR- FITC	L243	BD	#555811
HLA DR- PE- 128	L243	BD	#347401
IL23R- PE	218213	RDSys	#FAB14001P
P2X7- APC	L4#12	AG Nolte	own
sIgD- PE	IA6-2	Biolegend	#348203
HELIOS- Alexa 488	22F6	Biolegend	#137204
IFNγ- Alexa 488	4S.B3	eBioscience	#11-7319-71
IL-6- APC	8C9	Immuno Tools	#21670066
IL-10- PE	JES3-9D7	eBioscience	#12-7108-82
IL17A Alexa 647	eBio64DEC17	eBioscience	#51717941
TNFα- Alexa 488	MAb11	eBioscience	#11-7349
IFNγ- PacBlue	4S.B3	eBioscience	# 57-7319-71

Reagents

Cell stimulation	*Clone*	*Company*	*Catalog Number*
anti- CD3	OKT3	Bioxell	#BE00001-2
anti- CD28	37407	R&D	#MAB342
Ionomycin		Sigma Aldrich	#I0634
Phorbol myristate acetate (PMA)		Calbiochem	#524400
Live/dead fixable dead cell kit		Invitrogen	#L34960
HDMAPP		Echelon	#I-M055

Cell culture	*Company*	*Catalog number*
Brefeldin A	eBioscience	#00-4506
eFluor670 cell proliferation dye	eBioscience	#650840-90
Trypan blue solution, 0.4%	Sigma Aldrich	#T8154
Vybrant CFDA SE cell tracer kit	Invitrogen	#V12883

Flow cytomety	*Company*	*Catalog number*
FACS Flow, 20L	BD Biosciences	#342003
Fixation buffer	eBioscience	#8222-49
Lysing solution	BD Biosciences	#349202
Permeabilisation buffer, 10x	eBioscience	#8333-56
FoxP3 Fix/Perm buffer 4x	Biolegend	#421401

Media and Buffers

Media and supplements	*Company*	*Catalog number*
Bovine serum albumin (BSA)	PAA	#K45-001
Dimethyl sulfoxid (DMSO) for cell culture	AppliChem	#A3672.0100
Dubelcco´s Phosphate Buffered Saline (PBS)	PAA	#H15-002
L-Glutamine, 200mM	Invitrogen	#25030-024
Lymphocyte Separation Medium	PAA	#J15-004
Penicillin/Streptomycin, 100x	PAA	#P11-010
RPMI 1640	Gibco	#61870
Serum, fetal bovine	Biochrom AG	#S0115
X-VIVO 15, serum free medium	Lonza	#BE 04-418F

Buffers and solutions	*Content*
FASC buffer	0.1% BSA, 0.2% NaN3 in1x PBS
MACS/Sorting buffer	0.5% human Serum, 2mM EDTA in 1x PBS
Standard Medium	FCS 10%, Pen/Strep 1%, L-Glu 2mM in RPMI
Freezing medium 1	RPMI 90% FCS 10%
Freezing medium 2	RPMI 40%, FCS 40%, DMSO 20%
Saponin Buffer	0.1% BSA, 0.3% Saponin in 1x PBS
Natrium EDTA 2mM	2mM Natrium EDTA in 1x PBS

Equipment

Equipment	*Company*
Benchtop refrigerated centrifuges	Hereaus, Beckman Coulter
FACS Aria cell sorter	BD Bioscience
FACSCanto	BD Bioscience
Freezers	Liebherr
Freezing container, Nalgene Cryo 1°C	Roth
Fridges	Liebherr
Incubator, IncuSafe	Thermo Scientific, Sanyo
Microscope	Zeiss
Neubauer improved chamber	Marienfeld
Nitrogen tank	MVE
Pipets	Eppendorf/Gilson
Racks	Roth
Sterile bank, class II standard	Thermo Scientific
Vortex-Genie 2	Scientific Industries
Waterbath	Eppendorf, GFL

Software

Software	*Company*
FACS DIVA	BD
SPSS 19.0	IBM

Consumables

Consumables	*Company*
Cryo tubes	Greiner
Eppendorf tubes	Eppendorf
FACS tubes	Sarstedt
LS/MS columns	Miltenyi Biotech
Parafilm “M”	Pechiney
Pipette tips	Sarstedt
Sterile surgical blades	Braun
Syringe filters	VWR
Tissue culture flasks	Sarstedt
Tissue culture plates	Sarstedt and Greiner

Donors

The cohort of JIA patients consists of children that were diagnosed and treated at three centres: at the Universitätsklinikum Eppendorf by Dr. Weißbarth-Riedel, at the Klinikum Bad Bramstedt by Dr. Nikolay Tzaribachev and at the Kinderkrankenhaus Altona by Dr. Sandra Breyer. Only children that were diagnosed with JIA according to the ILAR criteria were included in this study (Petty et al., 2004).

Since a definite diagnose of JIA can only be made if symptoms persist for six weeks, samples of children that presented for the first time with signs of arthritis were also collected and processed, but only included in the study after confirmation of the diagnose by the attending physician when the child was being reassessed. All participants in the study were informed about the details of the study and a written consent was obtained in each case from the parents or legal guardians. One tube of heparin blood was drawn coinciding with blood draw for the routine laboratory exams. Synovial fluid was collected in a syringe with no added agents from patients who underwent joint puncture for diagnostic or therapeutic purposes. Patients were excluded if they had an acute infection at the time of sampling or if they had suffered from other chronic or metabolic diseases in the past.

Blood from healthy donors was collected at the Kinderkrankenhaus Altona in Hamburg. Children undergoing orthopaedic surgery were chosen by the attending orthopaedic surgeons on the basis of not having any acute infection, chronic disease or malignancy. All samples were collected with signed consent of the children's parents or legal guardians.

Methods

Sample collection

Blood from patients and healthy donors was processed immediately in the lab for surface staining and isolation of peripheral blood mononuclear cells (PBMCs). For practical reasons, material for intracellular cytokine staining was left over night on the shaker at room temperature (RT) to be processed the next morning. Synovial fluid was centrifuged at 300 g (earth's gravitational acceleration) for five minutes. After aspiration and additional centrifugation at 450 g for five more minutes, the supernatant of the synovial fluid was frozen at -20°C. Synovial fluid cells collected after the first centrifugation were washed twice with PBS and counted. The total cell number was determined using a Neubauer counting chamber. Cells were processed as described below and unused material was cryopreserved.

Surface staining

Surface staining was performed on whole blood. For our study we designed antibody panels to analyse different receptors on the surface of leukocytes. The antibodies for each panel were pre-mixed and first tested with blood of healthy donors before staining the patients' samples. The antibody mixtures are described in Table 2. Fifty microliters (50 µl) of whole blood and the corresponding amount of the antibody mixture were incubated for 30 minutes at RT and subsequently lysed with 1 ml of BD Lysing solution for 10 minutes. Then the cells were washed and resuspended in 300µl of FACS buffer for analysis in a flow cytometer.

For leukocyte characterisation of synovial fluid, 200.000 cells of the synovial fluid were stained with the same antibody mixtures for 30 minutes at RT, then washed twice with PBS and resuspended in 300µl of FACS buffer. The samples were either left over night in the fridge to be measured with the flow cytometer the next day or analysed immediately. For all measurements the FACS Canto was used.

Intracellular cytokine staining on whole blood

Because we had only access to limited amounts of blood and we wanted to reduce sample manipulation to a minimum level, we chose a protocol using whole blood for the intracellular staining. For this, 100µl of heparine blood were stimulated with 50ng PMA and 1 ng Ionomycin in the incubator (37°C, 5% CO_2). After an incubation period of 30 minutes 3µg Brefeldin A was added to each sample to prevent exocytosis of cytokines. After a total incubation of 4 hours, 2µl of 2 mM Natrium EDTA was added to all samples to prevent cell adhesion to the FACS tubes. Following an incubation period of 15 minutes this surface staining against the γδ-TCR was performed and cells were incubated in the dark for another 30 minutes. After 10 minutes incubation time the live/dead staining was added to the samples. The blood was then lysed with 1ml of BD Lysing solution and incubated for 10 minutes. Cells were then washed twice with cold PBS at 450 g for 5 minutes. To permeabilize the cell membrane the lysed blood was incubated for 15 minutes with a 0,3% Saponin Buffer containing 0,1% BSA. After this, cells were stained for the surface markers CD3 and CD4 and the intracellular molecules of interest (IFNγ, TNFα, IL-17). We performed the CD3 and CD4 staining together with the cytokine staining to prevent the breaking of the antibody conjugate. In all setups we used an unstained and a fluorescence-minus-one staining as a control. Cells were incubated for another 30 minutes with the antibodies and then washed twice. Before measuring with the flow cytometer, cells were resuspended in 300µl of FACS Buffer.

Intracellular surface staining on PBMCs and synovial fluid cells

In case the intracellular cytokine staining using whole blood was not successful, the staining was repeated using isolated PBMCs. The following protocol was also used in all intracellular cytokine stainings of synovial fluid cells. At least 50.000 PBMCs or synovial fluid cells were suspended in ex-vivo media and incubated with 10ng PMA and 0,2ng Ionomycin for 5 hours. After 1 hour of incubation 2ng Brefeldin A was added to each sample. Next, the cells were stained with the antibody against the γδ-TCR and incubated for 30 minutes. After 10 minutes of incubation the live/dead staining was added. Following this, cells were washed and then incubated with the fixation buffer for 10 minutes. Then, cells were washed twice with permeabilization buffer and afterwards stained for the surface markers CD3 and CD4 and the intracellular cytokines IFNγ, TNFα and IL-17. After an incubation period of 30

minutes, the cells were again washed with permeabilization buffer and resuspended in 300 µl FACS Buffer for analysis in a flow cytometer.

Isolation of mononuclear cells from the peripheral blood

From the remaining blood of patients and healthy controls we isolated PBMCs. For this, blood was diluted 1:3 with PBS and layered carefully onto 10 ml of lymphocyte segregation medium following a centrifugation of 800 g for 30 minutes at RT. Due to their size, shape and density PBMC fall through the lymphocyte segregation medium and form a layer in the interphase between plasma and segregation media. Cells were collected from the layer and washed twice with PBS. Without counting, cells were frozen immediately. The whole procedure was performed under sterile conditions.

Sample freezing

The remaining PBMCs and synovial fluid cells, which were not used for the phenotypical analysis, were frozen. For this, cells were centrifuged, resuspended in 0,5 ml of freezing media 1 and placed into cryo tubes. After slowly adding 0,5 ml of freezing media 2, the freezing tubes were stroed into a freezing container, allowing rapid freezing (approximately -1 °C/minute). All frozen samples were kept in a -80°C freezer.

Flow cytometry

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data of single cells. After staining, cells pass the light source they scatter light. Electrons in the fluorochromes are excited to a higher energy state and emit light. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes. Different detectors, which use optical long pass and band pass filters, receive light. Scattered and emitted light from cells is converted to electrical pulses by the optical detectors (photomultiplier tube, PMT). After the different signals or pulses are amplified they are processed and converted to digital information that can be graphically plotted.

Depending on the number of lasers and detection filters, different fluorochromes can be measured in parallel. Our flow cytometer (FACS Canto, BD biosciences) is equipped with three lasers and eight filters for detection of different fluorochromes, which means that we can use up to eight different antibodies-linked fluorochromes for cell characterization. In that way flow cytometry allows performing a detailed and precise phenotypical characterization of single cells found in the blood and synovial fluid of JIA patients. For all analysis FACS Diva software (BD Biosciences) was used.

Statistical analysis

For all statisitcal anlysis, outliers from different study groups (e.g. all healthy donors, all untreated JIA patients, all polyarticular JIA patients) were identified using the Grubbs' test. Outliers were exluded from statistical analysis but are shown in the graphs marked in parenthesis. Paired Student's t-test was used to compare the frequency of desired cell populations in peripheral blood and synovial fluid. To compare the frequency of different cell subsets between healthy donors and JIA patients we used the unpaired Student's t-test. The equality of variances was tested using the Levene's test. To analyse differences in the frequency of cell populations of healthy donors and different subtypes of JIA we used an ANOVA analysis. Correlation analysis was performed using a linear correlation analysis and by calculating the Pearson's correlation coefficient (r). We used the software SPSS 19.0 for all statistical analysis. Differences were considered significant if $p \leq 0.05$.

RESULTS

Ethics approval

A first step for the initiation of a project involving human samples was the approval of the local ethics committee. We designed a study protocol containing the names of participating doctors, patients' selection criteria, detailed description of planned experiments (phenotypical characterization, functional studies and in-vitro experiments of immune cells) and the information and consent forms for the patients' parents (Appendix 1: Information for parents and Appendix 4: Consent form). The study protocol was sent to the local authorities, in this case the Ärztekammer Hamburg, for ethics approval. After a first review the major objections concerned the information sheet for the patients arguing that children could not understand the information provided for the parents. Therefore, I designed a separate information sheet for children including a comic (Appendix 2: comic for children) explaining the procedure, which was attached to the parents' information sheet. Since different age groups of children participated in this study, the information was adapted to suit three age groups (Appendix 3: Information for children). After further review the committee had no more concerns and approved the study definitely in May 2011 (Appendix 5: Ethics approval).

Patient cohort

Fifty-two patients were enrolled in the study between the approval of the study and September 2011. All patients were assigned an alphanumeric code, which was kept during follow-up appointments. The collective of JIA patients had a mean age of 11,47 ± 4,84 years, ranging from 3 to 23 years. The subtypes of JIA were distributed as follows: 20 patients with polyarthritis, 17 with oligoarthritis, 11 with enthesitis-related arthritis, 3 with systemic arthritis and 1 patient with psoriatic arthritis (Appendix 6: Patient list). Patients were further divided according to their treatment (Figure 6): Patients not receiving any medication were combined with those who were treated exclusively with non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen or naproxen. Patients that were treated with steroids were placed into a separate group, since cortisone, even if applicated intra-articularly, has systemic

effects on the immune system. ‘DMARDS’ included all patients receiving disease-modifying drugs such as methotrexate and sulfasalazine. In the group ‘biologics’, patients were treated with genetically engineered proteins inhibiting specific cytokines such as TNFα and IL-1. The separation into the described treatment groups was based on the strategy of treatment: NSAIDS are the first-line treatment of JIA, DMARDS are used when NSAIDS fail and biologic agents only have a role in severe forms of JIA. When patients received combined medications from different groups, the strongest medication was used to identify the medication group (e.g. patients that received ibuprofen and methotrexate were placed in the group of ‘DMARDS‘).

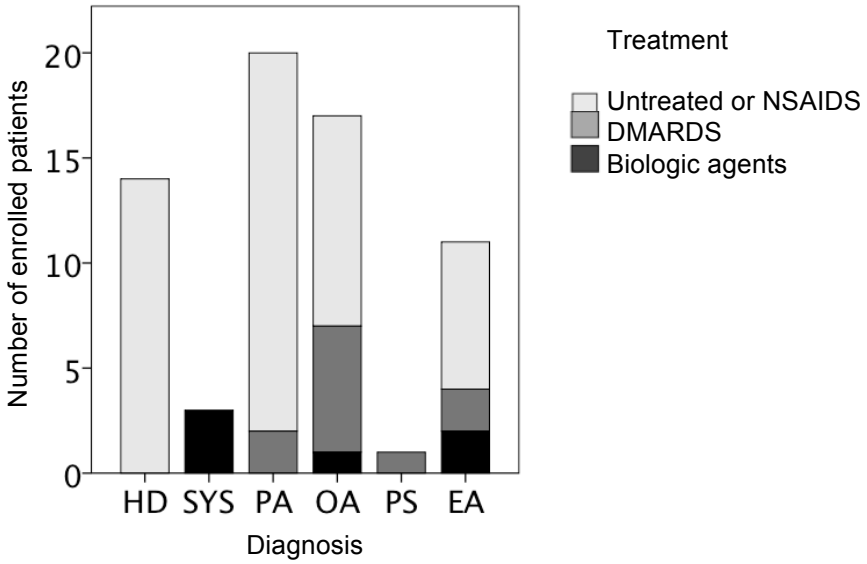

Figure 6 Frequency of enrolled patients.
Shown in the figure is the frequency of patients in each subgroup of JIA. Within the subgroups we further differentiated into different therapy plans.

Paired samples of peripheral blood and synovial fluid could be obtained from nine patients, seven with oligoarthritis and 2 with polyarthritis. In addition, fifteen control patients were collected with a mean age of 11,00 ± 3,42 years (range 0 to 16 years). One control sample had to be excluded, because the child had strikingly increased inflammation markers and, therefore, could not be used as a healthy donor.

Development of antibody panels to identify leukocyte subsets

To characterise different cell subsets and their activation and effector status in the peripheral blood and the synovial fluid of patients we designed antibody panels. The setup of each cocktail is shown in Table 2. Any antibody integrated in the panel was first titrated to determine the exact dilution yielding a discriminatory staining and to reduce spectral overlap with other fluorochromes. Data compensation of all FACS stainings was conducted thoroughly to avoid false positive stainings. In all cases analysed the quality of the sample was sufficient to ensure reliability.

Table 2 Antibody panels for the phenotypic analysis of peripheral blood and synovial fluid.

Fluorochrome label	V510	V450	FITC	PE	PE-Cy5.5	PE-Cy7	APC	APC-Cy7
Subsets	CD45	CD14	CD4	γδ-TCR	CD3	CD19	CD56	CD16
Effector T cells	CD4	CD8	CD28	γδ-TCR	CD3	CD45RA	CCR7	CD27
T cell activation	CD4	CD25	γδ-TCR	CD62L	CD3	-	CD69	CD27
Intracellular cytokines	Live/ dead	IFNγ	TNFα	γδ-TCR	CD3	-	IL-17	CD4
Chemokine receptors	-	CD4	CXCR3	CCR6	CD3	CCR4	CD161	CD27
Regulatory T cells	CD45	CD8	CD26	CD25	CD127	CD39	CD4	CD69
Ectoenzymes	-	CD8	CD38	CD73	CD3	CD39	P2X7	CD4
MAIT cells	CD4	CD8	γδ-TCR	IL23R	CCR6	CD45RA	CD161	CD27
Natural Killer Cells	-	CD14	CD57	CD161	CD3	-	CD56	CD16
B cells	-	CD20	CD38	IgD	CD3	CD19	CD4	CD27

Following the gating strategy shown in Figure 7, the subsets tube provided us with information about the relative frequency of all major leukocyte subtypes, namely granulocytes, monocytes and lymphocytes within CD45+ leukocytes. We further distinguished lymphocytes into B cells, TCRαβ+ T cells (CD4+ and CD8+), TCRγδ+ T cells, Natural Killer cells (CD56dim and CD56bright) and NKT cells.

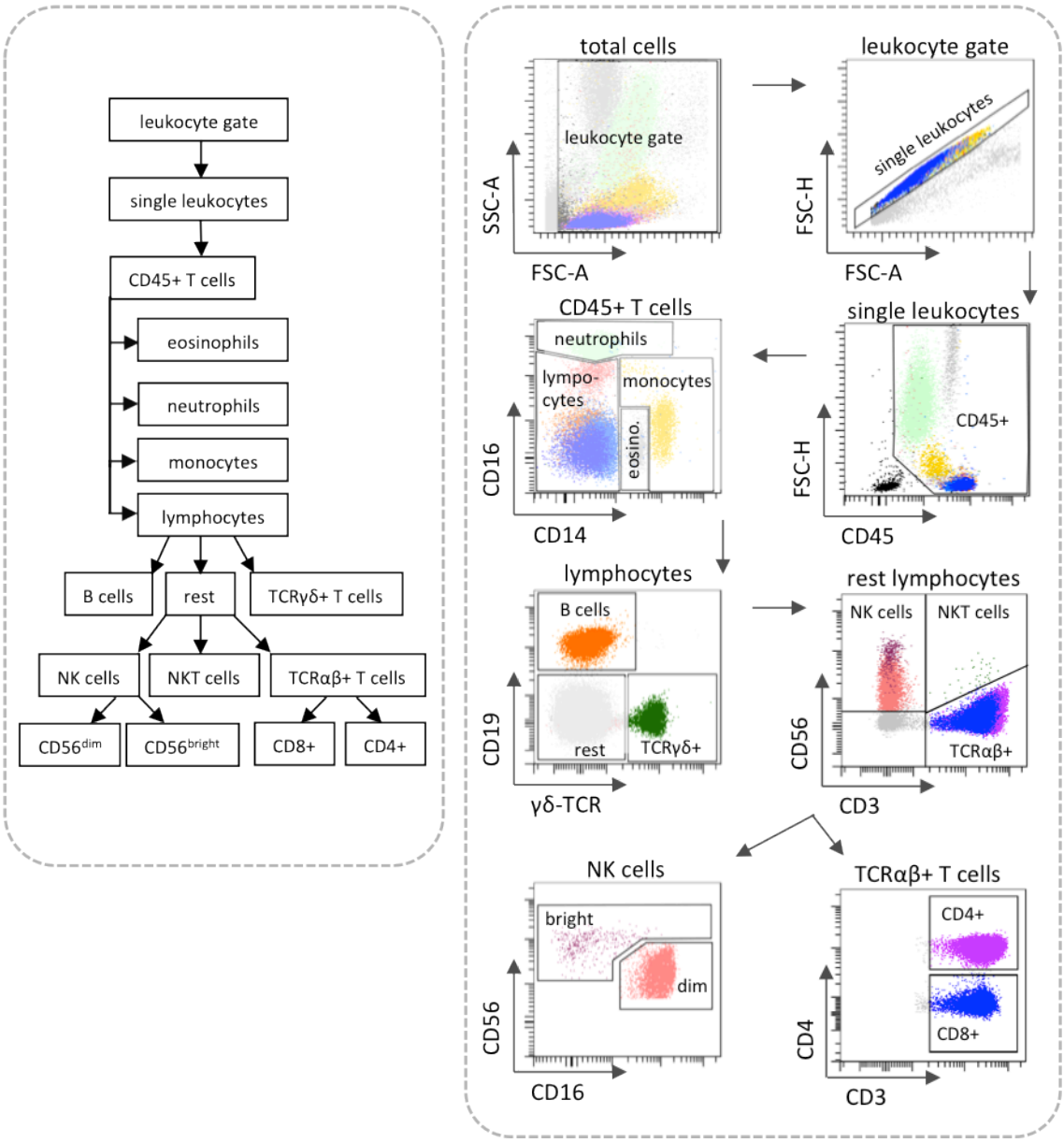

Figure 7 Human peripheral blood stained with the antibody panel "subsets".
Shown in the figure is the gating strategy to determine the relative frequency of major leukocyte subsets. On the left side, a schema illustrates the gating strategy, whereas on the right side representative dot plots of each gating step are shown. The title of each dot plots indicates the cells that are shown in the plot below. Granulocytes and monocytes were identified out of CD45+ leukocytes using the surface markers CD16 and CD14, respectively. Eosinophils were identified on the basis of their high autoflourecense in the V500 channel. The remaining cells were grouped as lymphocytes and could be further distingusihed into B cells, TCRαβ+ (CD4 + and CD8+) and TCRγδ+ T cells, Natural Killer cells and NKT cells by using the surface markers CD19, TCRγδ+ T cell receptor, CD4, CD3 and CD56. The expression level of CD56 together with the presence of CD16 distinguishes between the two subgroups of NK cells, $CD56^{bright}$ and CD56 Natural Killer cells.

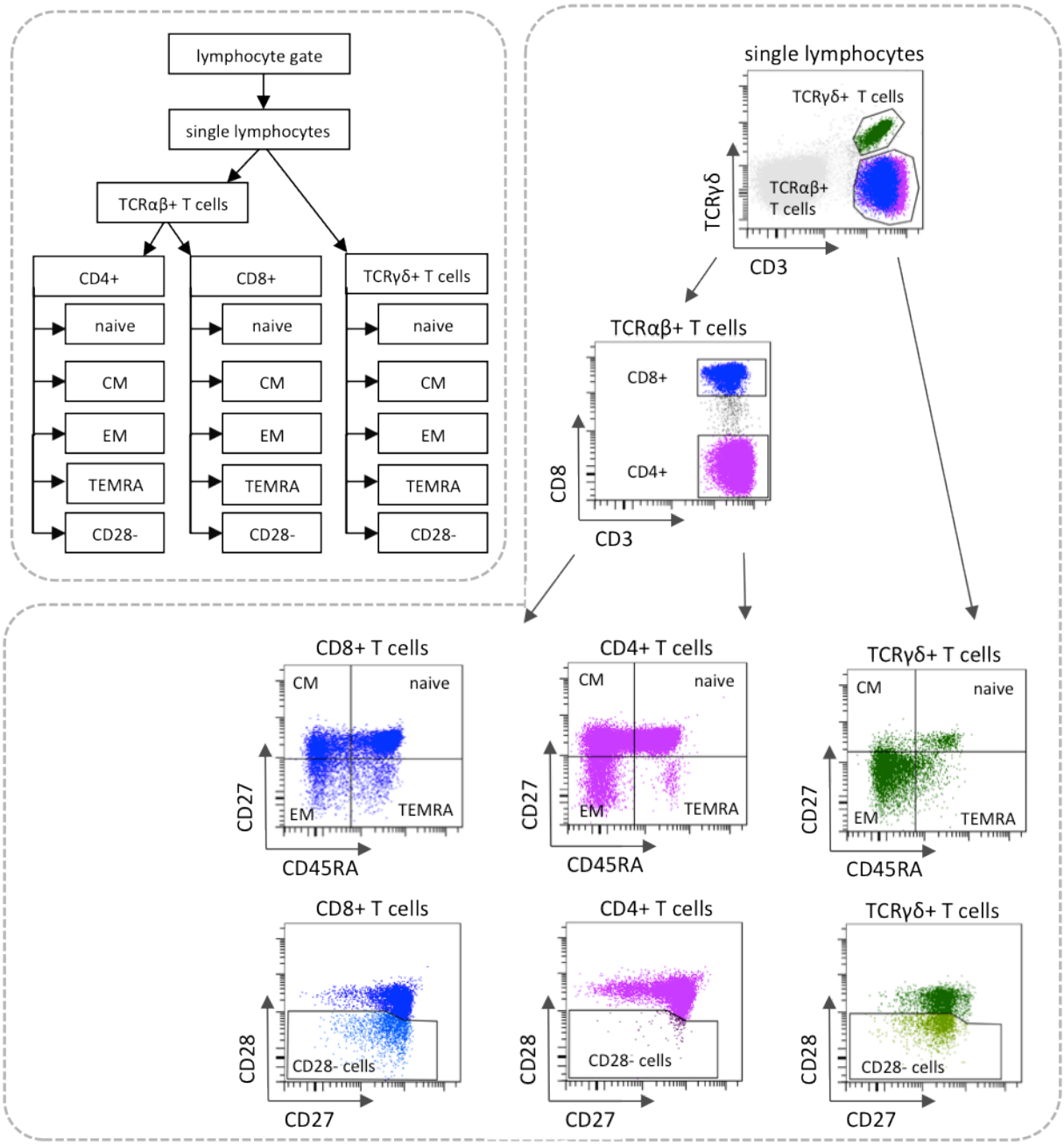

Figure 8 Human peripheral blood stained with the antibody panel "effector T cells".
Shown in the figure is the gating strategy to identify naïve and memory subsets of conventional TCRαβ+ and TCRγδ+ T cells. We first identified CD4+, CD8+ and TCRγδ+ T cells. Using the markers CD45RA and CD27, we identified naïve (CD45RA+ CD27+), central memory (CD45RA- CD27+), effector memory (CD45RA- CD27-) and TEMRA (CD45RA+ CD27-) T cells. Within CD4+ T cell, CD28- cells mark chronically activated cells.

The surface markers CD45RA, CCR7 and CD27 allowed us to determine the maturation level of lymphocytes. In the effector subsets panel, naïve T cells are characterised as CD45RA+ and CD27+/CCR7+, whereas memory T cells express the CD45RO isoform and only partly express CD27/CCR7. Although CCR7 is a better marker to distinguish between central memory (CCR7+) and effector memory

(CCR7-) T cells, it is downregulated during inflammation, while CD27 is a more stable marker. Thus, in our study we defined central memory cells as CD45RA- CD27+ T cells and effector memory cells as CD45RA- CD27- T cells (Figure 8). This combination of markers also identified memory subets of TCRγδ+ T cells. In combination with CD28 we were also able to identify chronically activated CD4+ CD28- T cells.

To characterise the activation status of TCRαβ+ and TCRγδ+ lymphocytes we used several markers (Figure 9): CD69 is an early activation marker, which is upregulated within few hours. Since CD69 is down regulated rather quickly and is only expressed temporarily, we used CD25 to identify cells that have been activated for a longer time period. CD25 is the α-chain of the IL-2 receptor. It is upregulated 12 to 24 hours after activation, correlating with the proliferation capacity of T cells. Due to a limited combination of antibodies in each panel, we used the “regulatory T cell” panel to identify CD4+, CD8+ and TCRγδ+ T cells that express CD25 (Figure 13). The “T cell activation” tube also included the marker CD62L, which is a cell adhesion molecule that allows leukocytes to leave the blood stream and enter into secondary lymphoid organs and into inflammation sites. CD62L is shed upon activation of leukocytes, and the absence of CD62L on the surface is an indicator of T cell activation or memory.

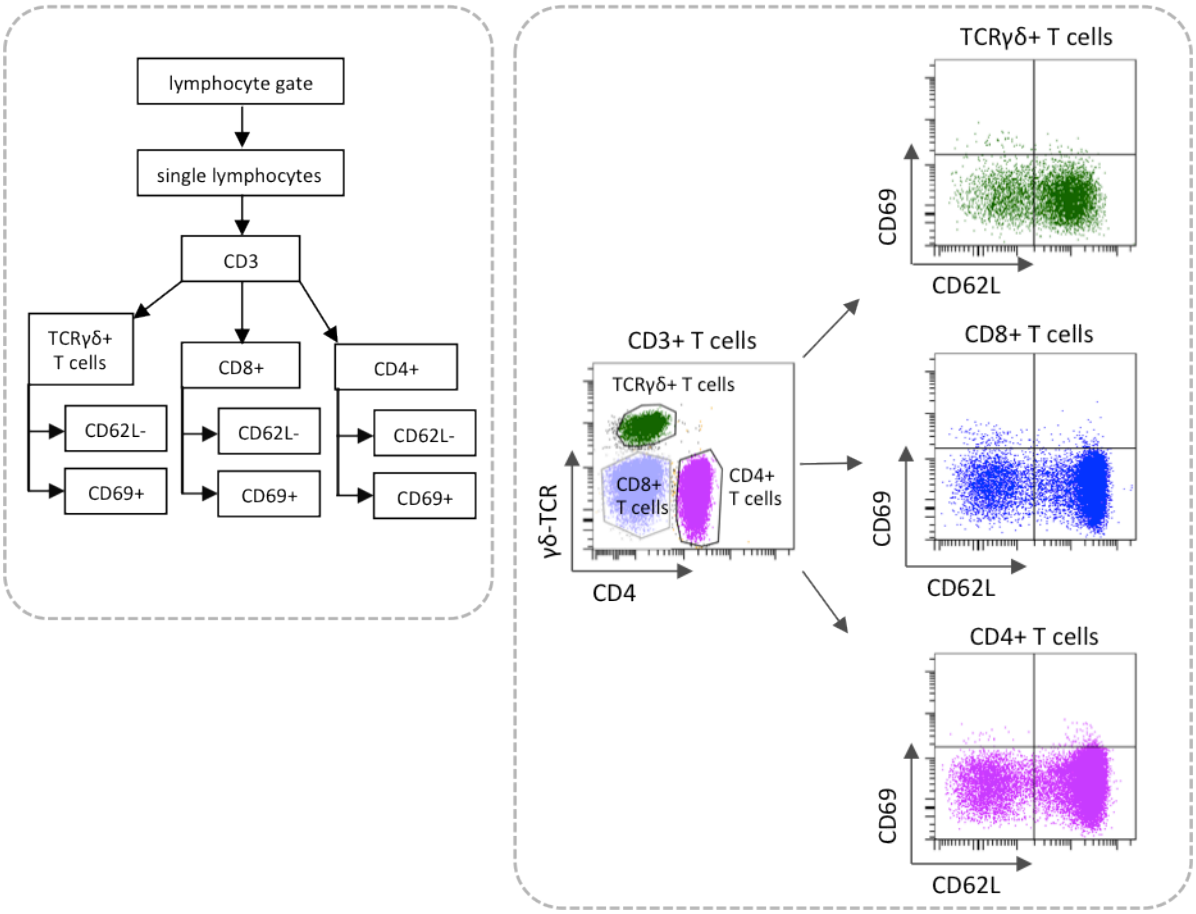

Figure 9 Human peripheral blood is stained with the antibody panel "T cell activation".
Shown in the figure is the gating strategy to identify CD69+ and CD62L- CD4+, CD8+ and TCRγδ+ T cells.

As shown in Figure 10, we analysed the production of IFNγ, TNFα and IL-17 in CD4+, CD8+ and TCRγδ+ T cells. Based on the production of IFNγ and IL-17 within CD4+ T cells we identified Th1 and Th17 cells, respectively. Alternatively, Th1 and Th17 cells can also be identified by the expression of a specific set of chemokine receptors (Sallusto and Lanzavecchia, 2009). In this study we identified Th17 cells as CCR6+ CCR4+ CD161+ CD4+ T cells and Th1 cells as CCR6± CCR4- CD161± CXCR3+ CD4+ T cells (Figure 11)

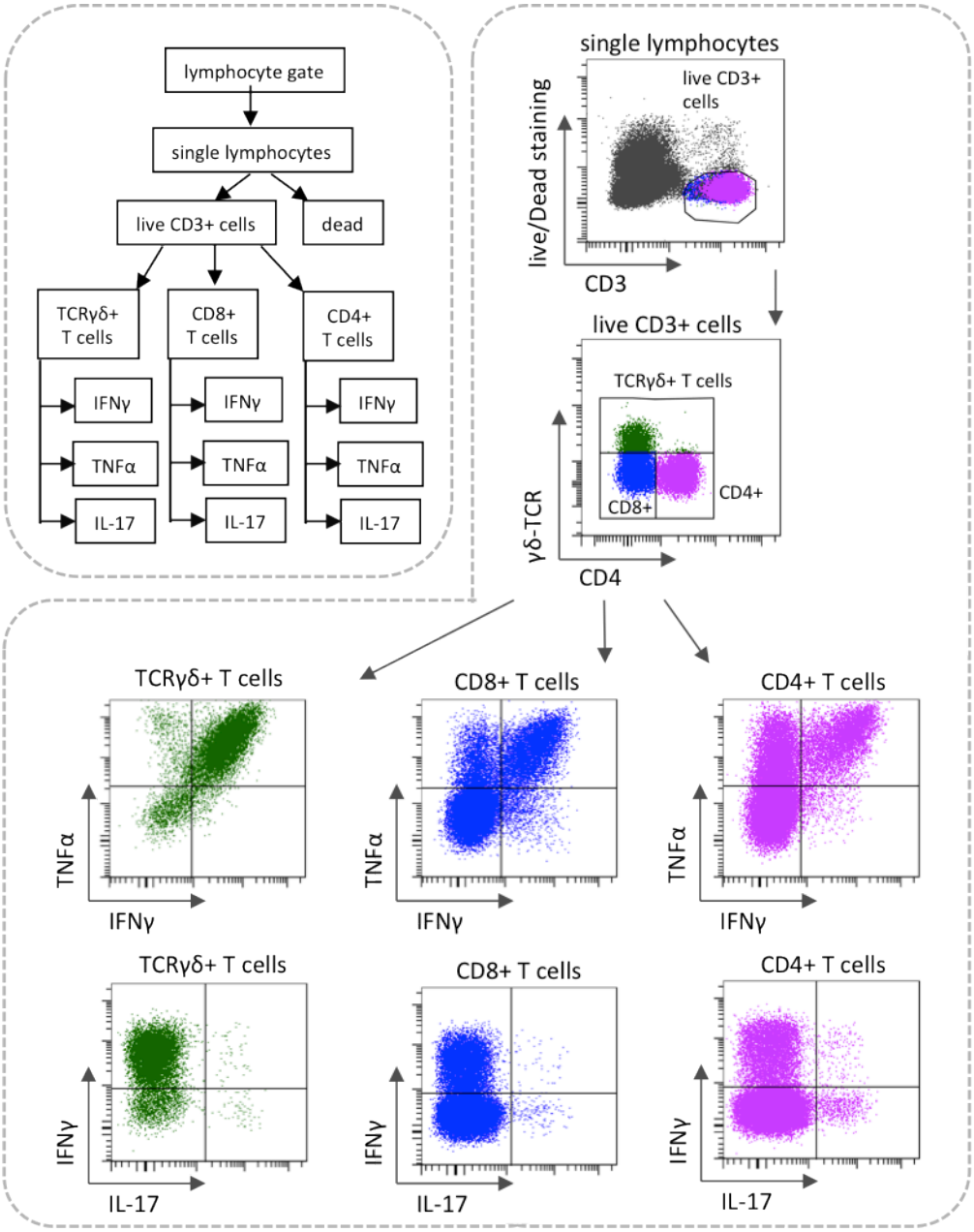

Figure 10 Intracellular cytokine staining of human peripheral blood.
Shown in the figure is the gating strategy to identify cytokine producing lymphocytes. Out of single, live CD3+ T cells we distinguished CD4+ T cells and TCRγδ+ T cells. Indirectly, we identified CD8+ T cells as CD4-, TCRγδ- CD3+ T cells. In all populations the production of IFNγ, TNFα and IL-17 was determined.

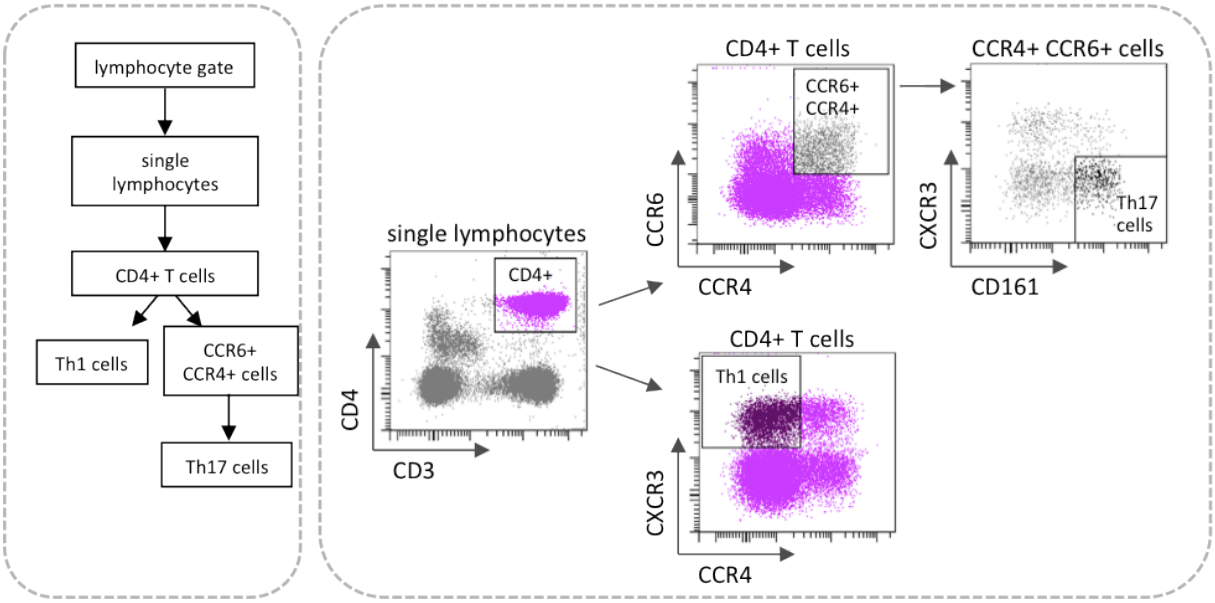

Figure 11 Human peripheral blood stained with the antibody panel "chemokine receptors". Shown in the figure is the gating strategy to identify Th17 cells (CD4+ CCR6+ CCR4+ CD161+ CXCR3-) and Th1 cells (CD4+ CXCR3+ CCR4- CCR6± and CD161±) solely by surface markers.

To confirm the assumption that Th1 and Th17 can be identified exclusively by surface markers, we performed a correlation analysis between the frequency of IFNγ and IL-17-producing CD4+ T cells and the percentages of T cells expressing characterisitc chemokine receptors for Th1 and Th17 cell, respectively (Figure 12). We performed a seperate correlation analysis for periperal blood and synovial fluid. In the peripheral blood of JIA patients and healthy donors we found a strong correlation between both strategies to identify Th1 cells (JIA patients: r= 0,84, p<0,001; healthy donors (HD): r= 0,85 p<0,01) and a medium correlation for the identification of Th17 cells (JIA patients: r= 0,49 p<0,001; HD: r= 0,42 p=ns). However, in the synovial fluid, we did not find a correlation. Thus, in inflammatory compartments the usage of intracellular cytokine staining seems to be more accurate to identify Th1 and Th17 cells.

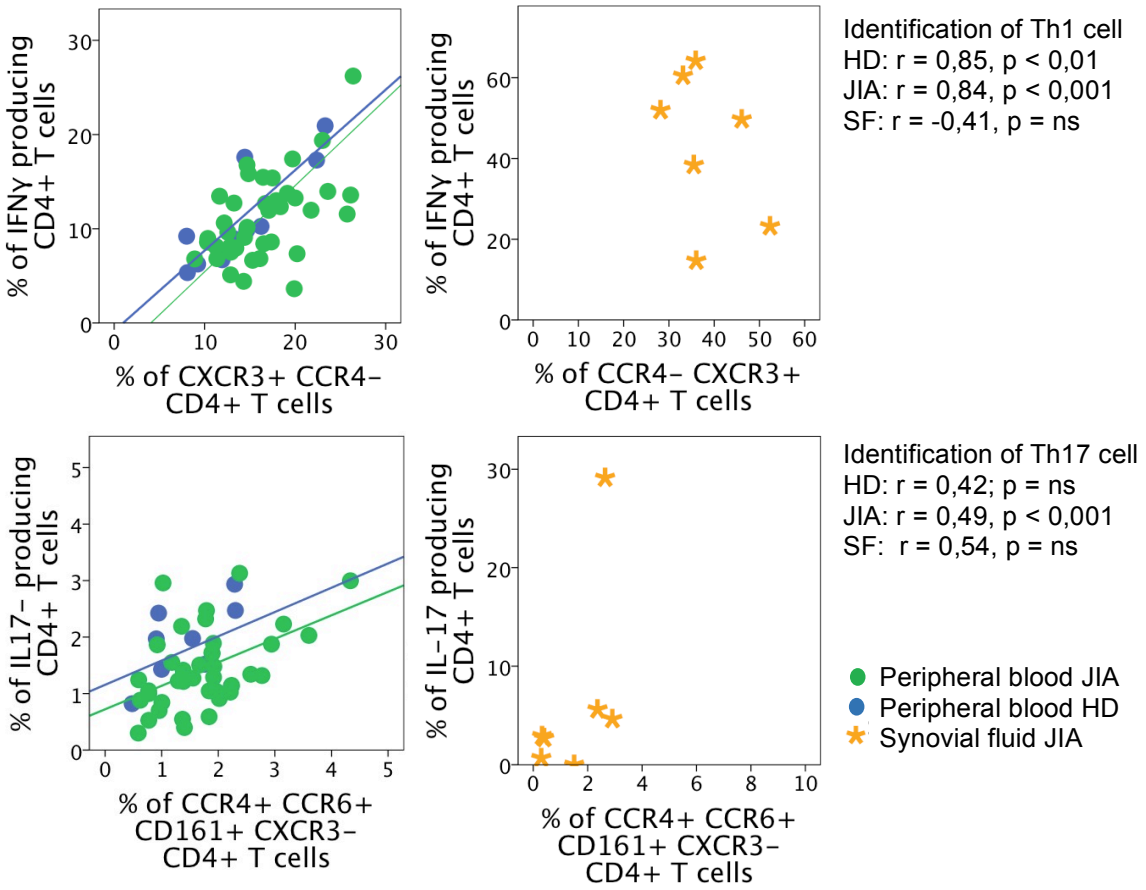

Figure 12 Correlation analysis of the frequency of Th1 and Th17 cells identified by specific chemokine receptors and by cytokine production.
The top panels show the correlation for the identification of Th1 cells in peripheral blood and synovial fluid while the lower panels display the correlation for the identification of Th17 cells. For the correlation analysis a Pearson product-moment correlation was performed.

With the regulatory T cells tube we gained insight into the frequency of CD4+ $CD25^{high}$ $CD127^{low}$ regulatory T cells, a cell type that can control excessive inflammation. Within Tregs we distinguished CD39+ and CD39- Tregs independently, because CD39+ Tregs show an increased capacity to suppress IL-17-producing T cells. Additionally, conventional CD4+ T cells that express CD39 may play a role in the pathogenesis of JIA (Moncrieffe et al., 2010), and are also considered in this tube. CD26 is a molecule that is expressed on conventional T cells and serves as a late activation marker. Furthermore, CD26 can bind the adenosine deaminase, which converts adenosine into inosine and, therefore, counteracts the anti inflammatory effect of CD39 and CD73, see “ectoenzyme tube”. The expression of CD39 and CD26 on CD4+ T cells is mutually exclusive.

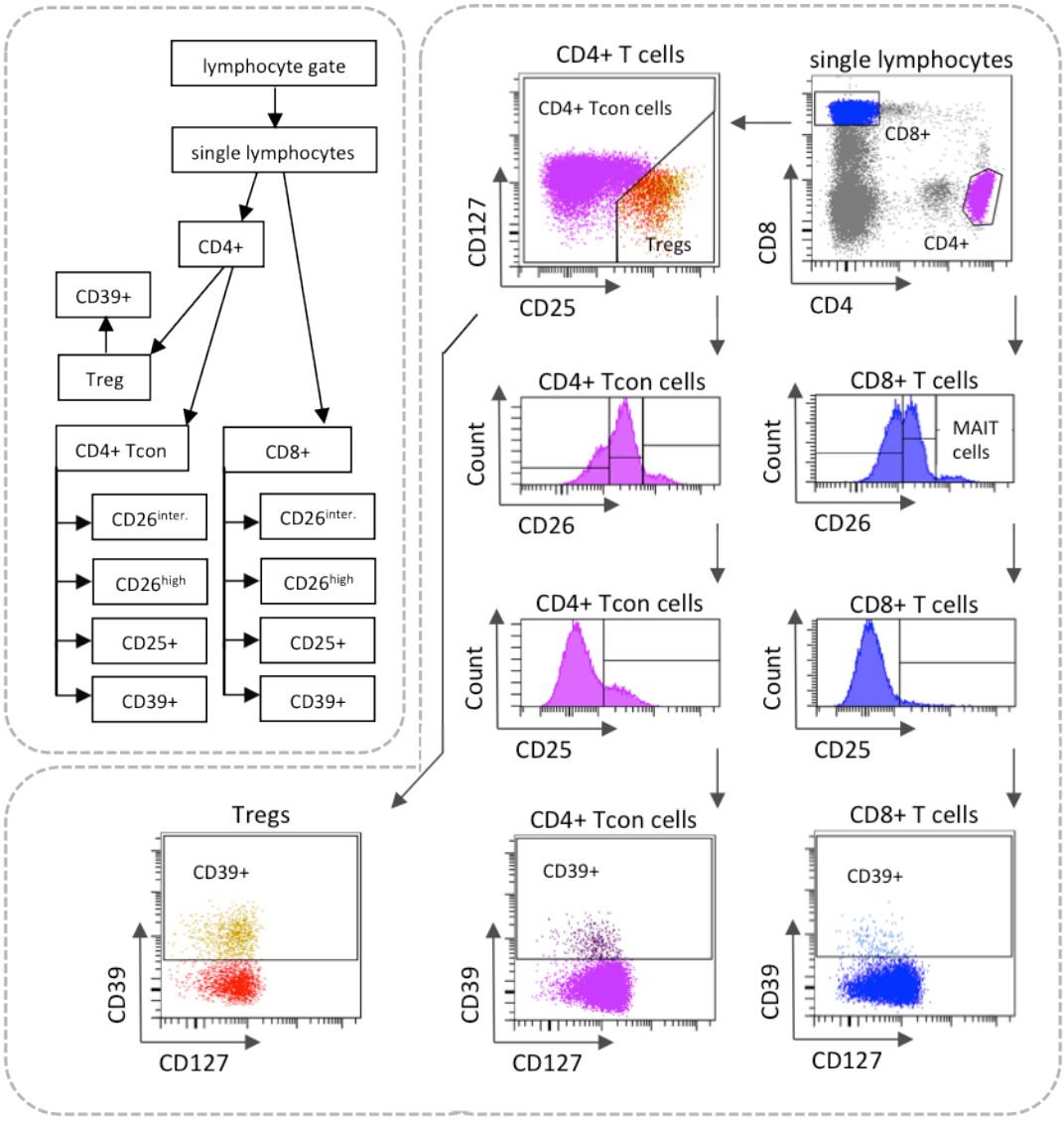

Figure 13 Human peripheral blood stained with the antibody panel "regulatory T cells". Shown in the figure is the gating strategy to determine the relative frequency of regulatory T cells. After gating single CD45+ cells we further distinguished into CD4+ and CD8+ lymphocytes. Out of all CD4+ lymphocytes, Tregs were identified as CD25+ and CD127low T cells. Two further subsets of Tregs were differentiated using the CD39 molecule. Also shown is the frequency of CD39 and CD26 expressing conventional CD4+ and CD8+ lymphocytes. Additionally, we used this antibody panel to identify MAIT cells, namely as CD26high CD8+ T cells.

CD39 and CD73 are ectoenzymes that are expressed on murine and human Tregs (Mandapathil et al., 2009). They cooperate in the elimination of the proinflammatory signal ATP. CD39 converts ATP into ADP and AMP, which then can be metabolized into adenosine by CD73. Adenosine has an anti-inflammatory function that leads to decreased T cell proliferation and production of IL-2 and IFNγ. Failure of the ATP breakdown could contribute to persisting inflammation in the joints of JIA patients.

For this study we analysed the expression of CD39 with the “regulatory T cell panel” whereas the frequency of CD73+ T cells was evaluated with the “ectoenzyme panel” (Figure 14). CD38 is an ectoenzyme with various functions. It is expressed as a late activation marker on T lymphocytes and serves as a differentiation marker on B cells. Furthermore, it catalyzes the metabolism of cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate, which are both essential regulators of intracellular Ca^{2+} homeostasis. It is also considered in the panel “ectoenzymes”.

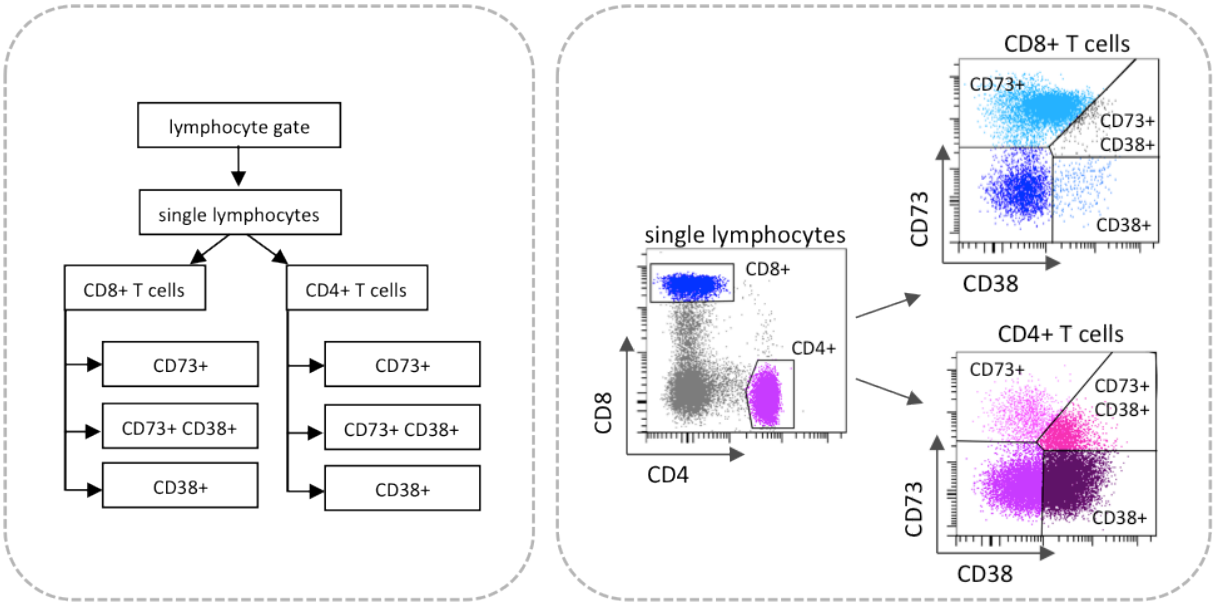

Figure 14 Human peripheral blood stained with the antibody panel “ectoenzymes”.
Shown in the figure is the gating strategy to determine the frequency of CD4+ and CD8+ T cells that express the ectonucleases CD38 and/or CD73.

Recently identified MAIT cells are an interesting cell subset that has been found in target organs of inflammatory diseases. MAIT cells express an invariant α-chain of the TCR (Vα7.2) and are overwhelmingly CD8-positive. Only a small proportion of MAIT cells are CD4- and CD8- double negative. They express high levels of CD161 and CD26. For this study we developed two strategies to identify MAIT cells: The first population were CD8+ CCR6+ CD161high T cells, which we identified with the MAIT panel (Figure 15). With the data of the regulatory T cell panel (Figure 13), we described the second population as CD8+ $CD26^{high}$ T cells. Due to our combination of antibodies in both panels we were not able to identify double negative MAIT cells.

CCR6 and CD161 identify T cells with the potential to produce IL-17. In the MAIT panel we combined these markers with a γδ-TCR antibody to find out if CCR6 and CD161 might also be suitable to identify IL-17-producing TCRγδ+ T cells, as reported for murine TCRγδ+ T cells. However, we saw no correlation between the frequency of IL-17-producing TCRγδ+ T cells and TCRγδ+ T cells the express CCR6 and CD161 (data not shown).

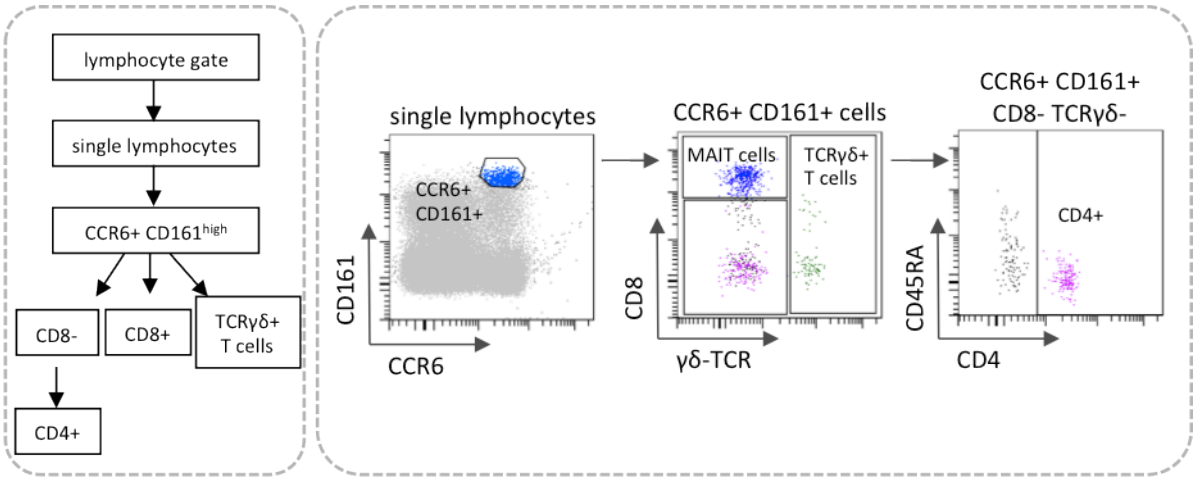

Figure 15 Human peripheral blood stained with the antibody panel "MAIT cells".
Shown in the figure is the gating strategy to identify CCR6+ CD161+ T cells. The gate to identify CCR6+ CD161+ cells was set according to the expression in CD8+ T cells. Out of all CCR6+, CD161high cells we identified those which express CD4, CD8 or the TCRγδ+CR.

Since we used two ways of identifying MAIT cells, we wanted to analyse the correlation of both gating strategies with each other. Since we saw that chemokine receptors are down regulated in the synovial fluid, we separated blood and synovial fluid samples for this correlation analysis. We could show that the frequency of CCR6+ CD161+ CD8+ T cells correlates very well with the frequency of CD8+ $CD26^{high}$ T cells (HD: $r = 0,97$, $p < 0,001$; JIA patients: $r = 0,95$, $p < 0,001$)(Figure 16).

Since there has been data suggesting that the frequency of MAIT cells increases with age, we also wanted to analyse the frequency of MAIT cells according to age of healthy donors and JIA patients. Figure 16 shows that the frequency of MAIT cells increased with age. The analysis between the frequency of $CD26^{high}$ CD8+ T cells and the age showed a medium correlation in JIA patients ($r = 0,51$, $p < 0,001$). In healthy donors we did not observe a significant correlation possibly due to the restriced sample size ($r = 0,23$, p = ns).

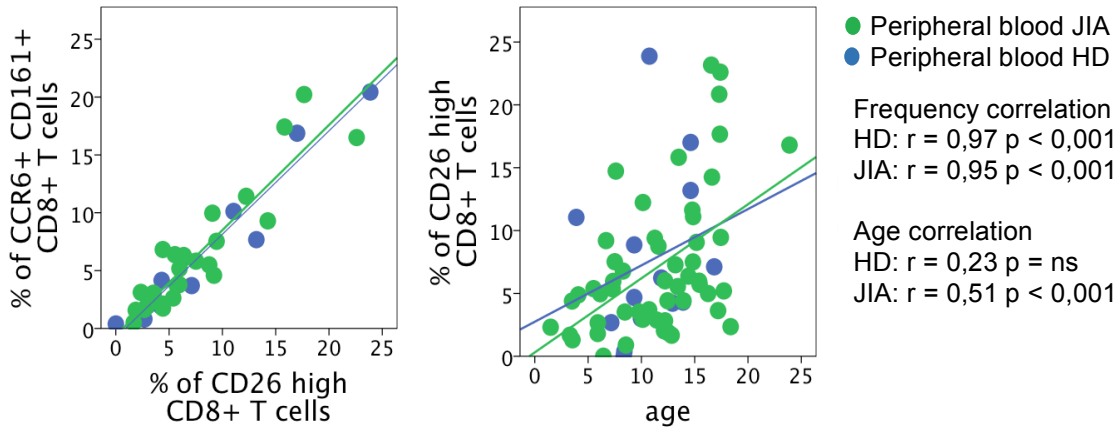

Figure 16 Correlation analysis of MAIT cells.
MAIT cells were either identified as CD8+ CCR6+ CD161+ T cells or as CD8+ CD26high T cells. Using blood samples we compared both strategies to identify MAIT cells and performed a linear regression analysis (left panel). The right panel shows the age correlation of the frequency of MAIT with age of HD and JIA patients.

The NK tube gave us information about the maturation status and the phenotype of NK cells. NK cells are defined as CD3- CD56+ cells, and the level of expression of CD56 identifies two distinct subsets: $CD56^{dim}$ (CD16+) and $CD56^{bright}$ (CD16-) NK cells. In human blood, more than 90% of the NK cells belong to the CD56dim subset, and deviation of this percentage may reflect disease status or response to treatments (Bielekova, 2006). NK $CD56^{bright}$ cells are interesting in the context of autoimmune diseases since they show regulatory functions. The markers CD161 and CD57 identify different maturation levels of NK cells. CD161 expression marks early innate NK cells whereas CD57+ NK cells are highly mature (Figure 17).

B cells are an interesting study target because different autoantibodies (ANAs and RFs) are found in JIA patients. The presence of RF distinguishes between two subgroups of polyarthritis, whereas postive ANAs are associated with a more severe progression of disease in patients with oligoarthritis. With the B cell tube we gained insight into the maturation status of B cells and the frequency of plasma cells in the blood and synovial fluid (Figure 18). While CD19 and CD20 are present from the very beginning of B cell differentiation, surface IgD marks naïve B cells in the peripheral blood. Upon activation B cells up regulate CD38 and develop into memory B cells or plasma cells. Memory B cells have an IgM/IgA/IgG+ CD27+ CD38- phenotype whereas plasma cells highly express CD38 and CD27 but have lost CD20.

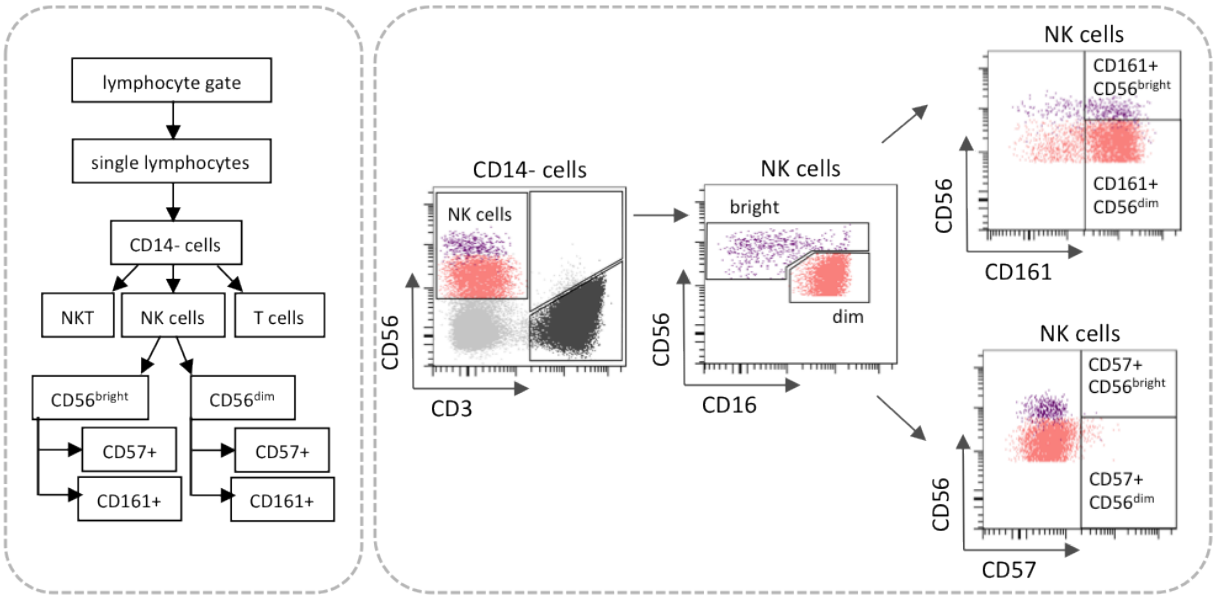

Figure 17 Human peripheral blood stained with the antibody panel "NK cells".
Shown in the figure is the gating strategy to identify NK cells. After excluding CD14+ monocytes we selected CD3- CD56+ cells and distinguished these into $CD56^{dim}$ and $CD56^{bright}$ NK cells. Expression of the maturation markers CD57 and CD161 are shown in $CD56^{dim}$ and $CD56^{bright}$ NK cells.

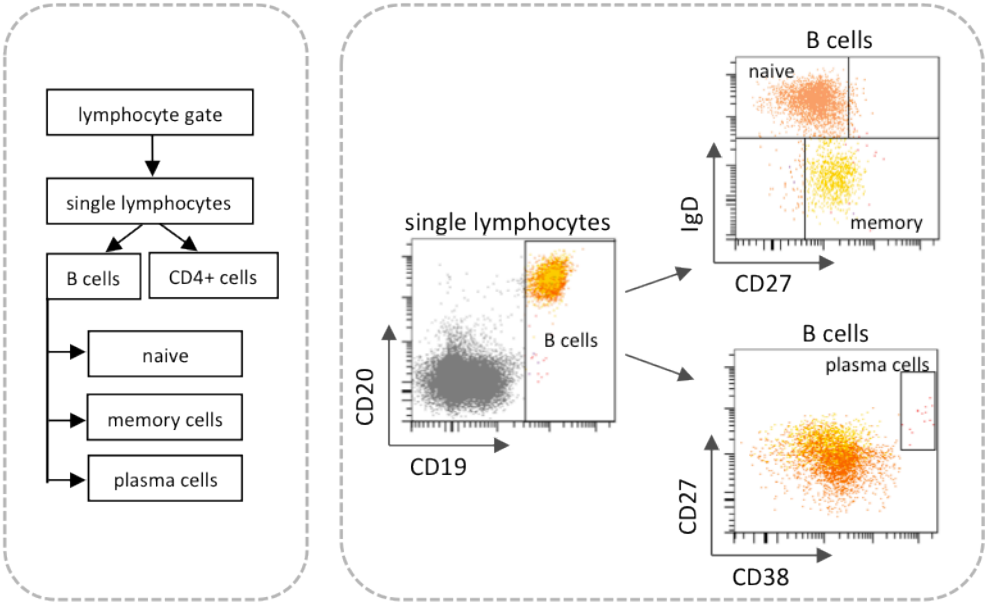

Figure 18 Human peripheral blood stained with the antibody panel "B cells".
B cells were identified as CD19+ lymphocytes. Within the B cell population we evaluated the maturation status with the surface markers IgD and CD27. Naive B cells are IgD+ and CD27- whereas after antigen encounter CD27 is upregulated. Finally after B cells have undergone class switch IgD is not expressed on the surface anymore. Plasma cells are matured B cells that produce antibodies and have lost the expression of CD20.

Immunophenotypic analysis of regulatory, inflammatory, memory and innate like lymphocyte subsets

In this study we performed a detailed analysis of a variety of immune cell subsets that might contribute to the pathogenesis and progression of JIA. An overview of all analysed leukocyte subsets is shown in Table 3. To evaluate activation, effector function, capacity of immune regulation and the influence of innate like immune cells, we divided analysed cell populations into seven groups: 1. leukocyte subsets, 2. maturational stage, 3. activation and inflammation, 4. effector function, 5. immune regulation, 6. ectoenzymes, 7. TCRγδ+ T cells and 8. MAIT cells.

Table 3 List of all anaylsed leukocyte subsets examined by flow cytometry.
Of note, regulatory CD4+ T cells are excluded from the populations marked with a *. Th1 and Th17 cells were identified by the expression of a set of chemokine receptors. **Th1= CD4+ CXCR3+ CCR4- T cells. ***Th17= CD4+ CCR6+ CCR4+ CXCR3- CD161+ T cells

1. Subsets	2. Maturational stage	3. Activation and Inflammation	4. Effector function
neutrophils	CD4+ naive T cells	CD4+ CD69+ T cells	CD4+ IFNγ-producing T cells
monocytes	CD4+ central memory T cells	CD4+ CD25+ * T cells	CD4+ TNFα-producing T cells
lymphocytes	CD4+ TEMRA T cells	CD4+ CD62L- T cells	CD4+ IL-17-producing T cells
NK cells	CD4+ effector memory T cells	CD4+ CD28- T cells	Th1** cells
TCRαβ+ T cells	CD8+ naive T cells	CD8+ CD69+ T cells	Th17*** cells
(CD4+ and CD8+)	CD8+ central memory T cells	CD8+ CD25+ T cells	CD8+ IFNγ-producing T cells
TCRγδ+ T cells	CD8+ TEMRA T cells	CD8+ CD62L- T cells	CD8+TNFα-producing T cells
B cells	CD8+ effector memory T cells		CD8+ IL-17-producing T cells
	naive B cells		
	memory B cells		
	plasma cells		
	$CD56^{dim}$ NK cells		
	$CD56^{bright}$ NK cells		
	CD57+ $CD56^{dim}$ NK cells		

5. Regulation	6. Ectoenzymes	7. TCRγδ+ T cells	8. MAIT cells
Tregs	CD4+ CD39+ * T cells	TCRγδ+ naive T cells	CD8+ $CD26^{high}$ T cells
CD39+ Tregs	CD4+ CD73+ T cells	TCRγδ+ central memory T cells	CD8+ CD161+ CCR6+ T cells
CD8+ CD28- T cells	CD4+ CD38+ T cells	TCRγδ+ TEMRA T cells	
$CD56^{bright}$ NK cells	CD4+ CD26+ T cells	TCRγδ+ effector memory T cells	
	CD8+ CD39+ T cells	TCRγδ+ CD69+ T cells	
	CD8+ CD73+ T cells	TCRγδ+ CD62L- T cells	
	CD8+ CD38+ T cells	TCRγδ+ IFNγ-producing T cells	
	CD8+ $CD26^{intermediate}$ T cells	TCRγδ+ TNFα-producing T cells	
		TCRγδ+ IL-17-producing T cells	

Analysis of paired peripheral blood and synovial fluid samples

Immune cell populations in the synovial fluid

The first step of the analysis was the comparison of leukocyte subsets in peripheral blood and the target organ, the synovial fluid of inflamed joints. In the synovial fluid we found a variable frequency of monocytes and neutrophils (Figure 19). Within lymphocytes, we found an expansion of CD8+ T cells and a reduction of CD4+ T cells compared to peripheral blood. The frequency of TCRγδ+ T cells and NK cells varied greatly within the patient cohort. Due to the low amounts of B cells in the synovial fluid we could use the frequency of B cells as an indicator of blood contamination that sometimes occured when small blood vessels are harmed during the puncture process.

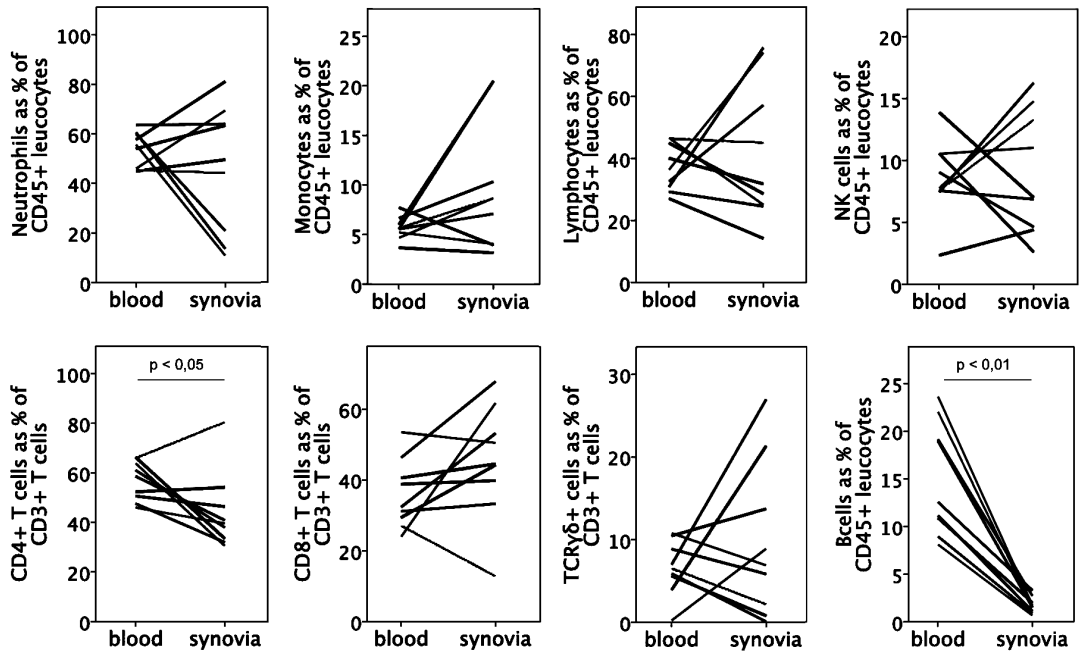

Figure 19 Frequency of leukocyte subsets in paired samples of peripheral blood and synovial fluid.
Peripheral blood and synovial fluid cells were stained with the "subsets" antibody panel and the frequency of each population was assessed from the CD45+ leukocyte population. Paired t-test was used to calculate statistical significance.

To illustrate individual differences, Figure 20 shows five representative examples of the composition of lymphocytes subsets between blood and synovial fluid. There are

several additional populations that are not shown in Figure 20, but that are part of the lymphocyte gate, namely CD3+ CD56+ (described as NKT cells) and CD3- CD56- cells. The gap to 100% is dependent on the size of these populations. Additionally, some synovial fluid samples also contained a lot of cell debri which mixed in with the lymphocyte gate.

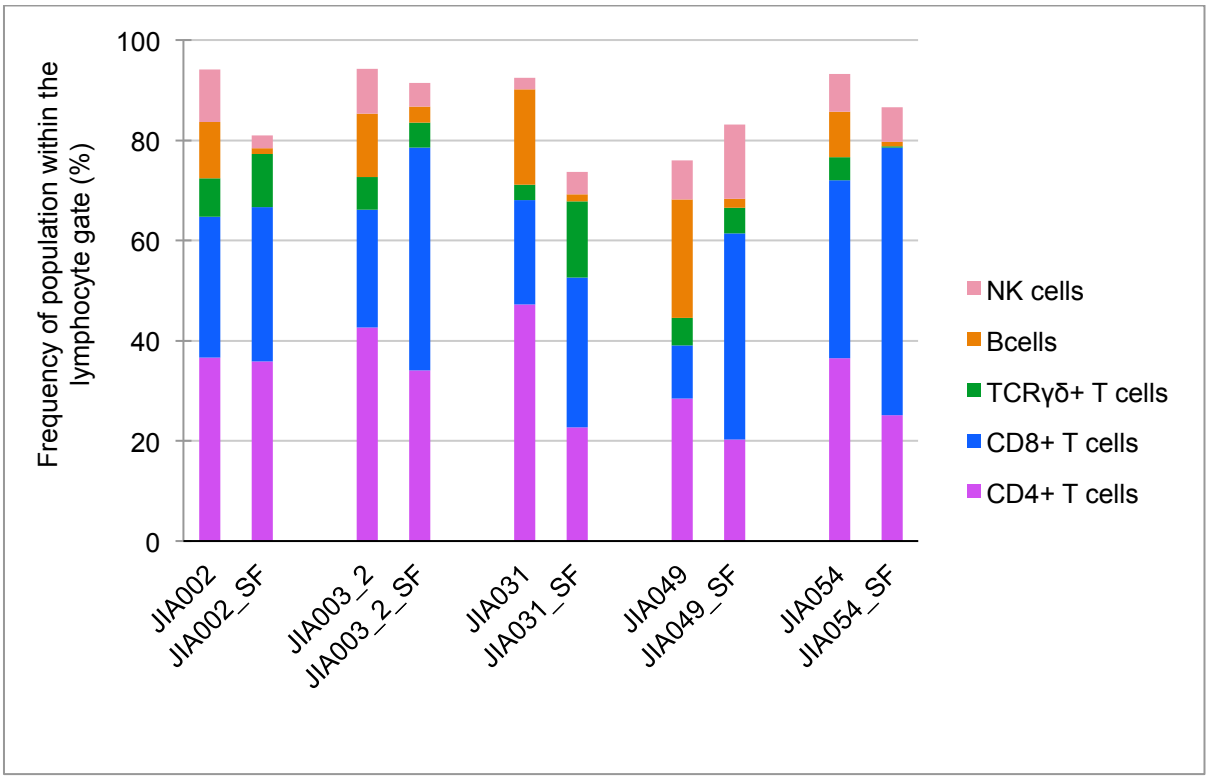

Figure 20 Composition of lymphocytes in paired samples of peripheral blood and synovial fluid of selected patients.
Shown in the figure is the frequency of CD8+, CD4+ and TCRγδ+ lymphocytes, B cells and NK cells related to the number of lymphocytes. The numbers are taken from the staining of peripheral blood and synovial fluid with the "subset" antibody panel.

Inflammatory, regulatory and memory cells accumulate in the synovial fluid

JIA is associated with chronic inflammation in the joints of patients. An inflammatory status can be characterized in several ways: By the composition of naive and memory lymphocytes, by the expression of classical inflammation markers and by the effector function of analysed lymphocytes. In a first step we wanted to analyse if the chronic inflammation in the joints of patients with JIA leads to an increase of more mature lymphocytes. Therefore, we identified naïve, central memory, effector

memory and TEMRA CD4+ and CD8+ T cells as well as maturation markers in NK and B cells.

In the T cell compartment of the synovial fluid we found a significant decrease in the frequency of naïve and a concomitant increase in the frequency of CD45RA- CD27+ central memory T cells. CD45RA- CD27- effector memory T cells were only significantly increased within CD4+ T cells although we also saw a slight increase within CD8+ T cells. The frequency of CD4+ TEMRA cells was similar within both compartments in the majority of the patients. Likewise most patients had similar amounts of CD8+ TEMRA cells in the peripheral blood and the synovial fluid. Interestingly, in two patients we saw an aberrant expansion of CD8+ CD45+ CD27- TEMRA T cells in peripheral blood (Figure 21).

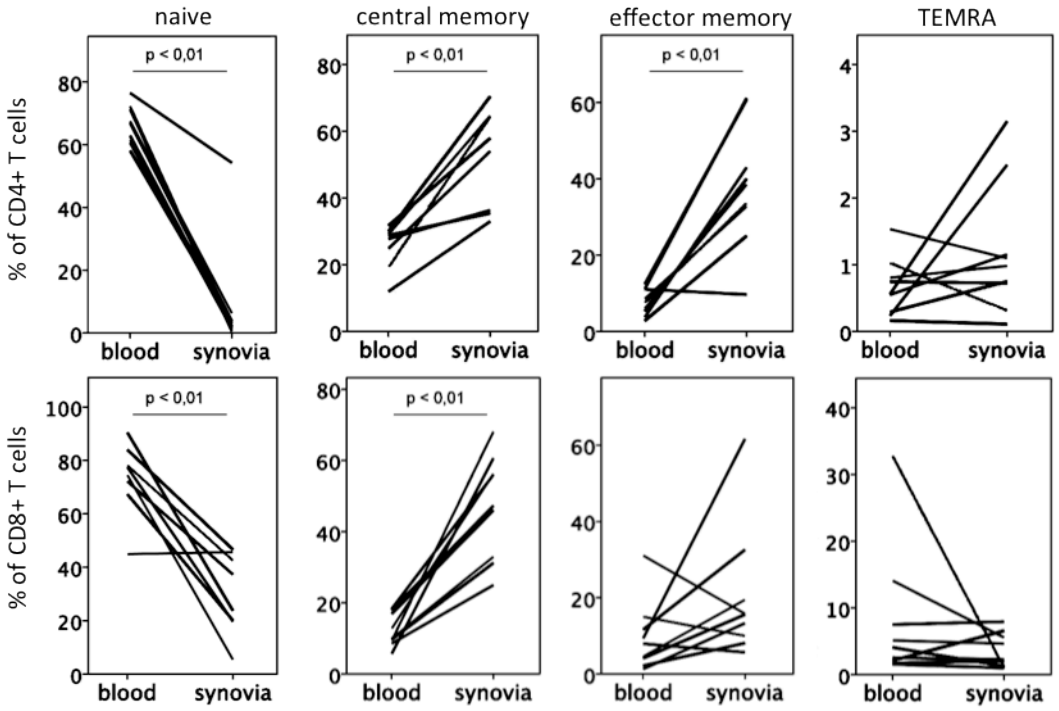

Figure 21 Frequency of CD4+ and CD8+ memory subsets.
Peripheral blood and synoval fluid cells were stained with the antibody panel “effector T cells”. The frequencies of the different maturational stages were set in relation to the total amount of CD4+ and CD8+ T cells. Differences were determined using a paired t-test.

The same pattern of decreased frequencies of naïve cells and an accumulation of memory cells was found in B cells. The majority of B cells in the synovial fluid were memory B cells whereas in the peripheral blood of the patients 60-80% of B cells

were naïve. Considering that the total amount of B cells in the synovial fluid is very small, this data indicates that naïve B cells are a very rare population in the synovial fluid.

Although the percentage of NK cells within lymphocytes was not generally different, we observed a dramatic increase in $CD56^{bright}$ NK cells, which resulted in an inverted ratio of $CD56^{bright}$ and $CD56^{dim}$ cells. Interestingly, the frequency of CD57+ $CD56^{dim}$ NK cells, which identify highly mature NK cells, was significantly decreased in the synovial fluid. It is the only matured cell subset that we did not find increased in the target organ.

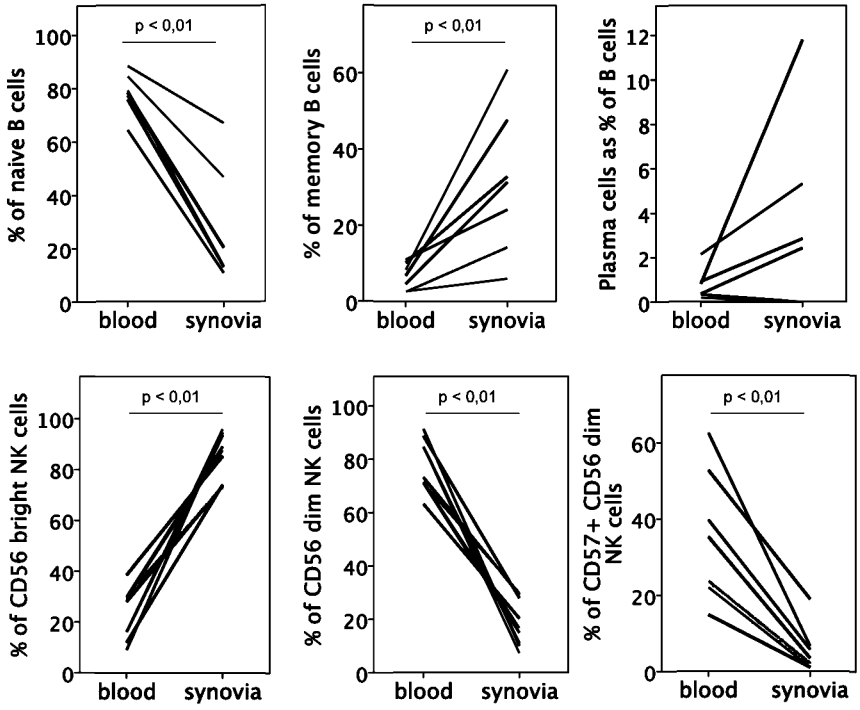

Figure 22 Frequency of B and NK cells expressing specific memory markers.
The maturation level of B and NK cells was determined as described before. Memory B cells and plasma cells were identified with the markers CD27, CD38 and surface IgD, whereas in NK cells we used the expresion of CD56, CD16 and CD57 to classify maturation levels. A paired t-test was used to calculate statistical significance.

Next we analysed the expression of a variety of activation markers. The ongoing inflammatory process in the joints was clearly reflected by an increase of activated T cells in the synovial fluid compared to peripheral blood. In the synovial fluid we found a significant increase in both CD69+ T cells representing recently activated cells and CD25+ T cells that have been activated for a longer period. Likewise, the frequency

of CD62L- T cells was increased at the site of inflammation. CD4+ T cells that have lost CD28 reflect a cell subset that has undergone repetitive stimulation and, therefore, could reflect chronic activation (Figure 23).

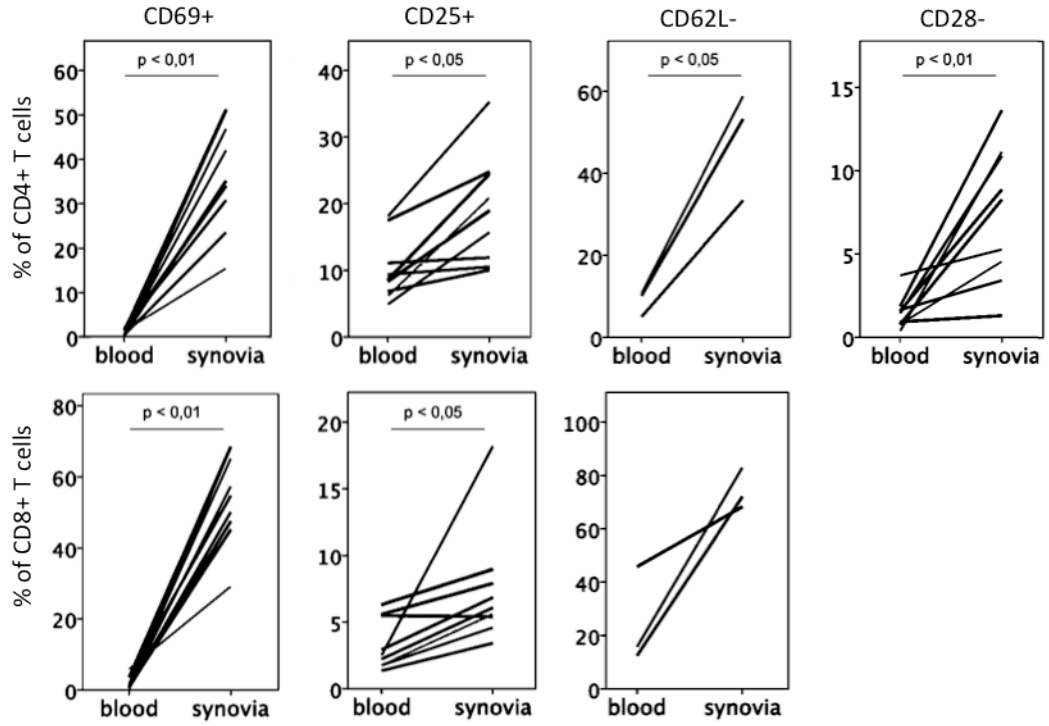

Figure 23 Frequency of CD4+ and CD8+ T cells expressing activation markers.
The data was taken from the stainings with the antibody panels “activated T cells” and “regulatory T cells”. The top panels show the frequency of CD4+ T cell populations whereas the lower panels display the frequency of activated CD8+ T cells. The frequencies of activated T cells was set in relation to the total amount of either CD4+ or CD8+ T cells. Significant differences are determiend with a paired t-test.

Proinflammatory cytokine production is characteristic of ongoing inflammatory processes. IFNγ and TNFα are long known as major cytokines related to synovial inflammation. The more recently identified cytokine IL-17 has also been associated with pleiotropic effects contributing to damaged cartilage and bone in inflamed joints of JIA patients. While we found a significant accumulation of IFNγ- and TNFα-producing CD4+ and CD8+ T cells in the synovial fluid of all patients, the frequency of IL-17-producing T cells was only increased in selected patients (Figure 24). Of note, one patient showed enormous amounts of IL-17 production not only in the CD4+ compartment but also in CD8+ T cells. We reassessed if this particular patient

also had an increased amount of CD8+ MAIT cells that may explain the raise of IL-17 production, but this was not the case.

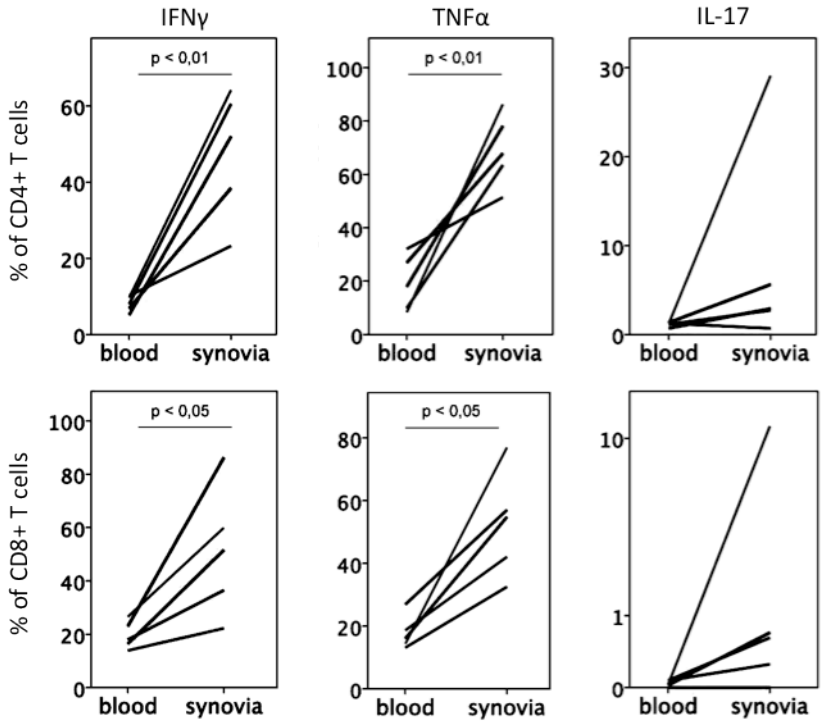

Figure 24 Frequency of T cells producing different cytokines.
Intracellular cytokine staining was performed on whole blood and synovial fluid cells. The top panel shows the frequency of IFNγ, TNFα and IL-17-producing CD4+ T cells, the lower panel shows the frequency of CD8+ T cells that produce the same cytokines. Significant differences were determined using a paired t–test.

Although it has been reported that regulatory T cells are increased in the joints of patients with JIA, there is no sufficient suppression of activated effector T cell during disease (de Kleer et al., 2004). We, therefore, wanted to expand the analysis of regulatory subsets in the synovial fluid including CD39+ and CD39- Tregs, CD28- CD8+ T cells and $CD56^{bright}$ NK cells. CD39+ Tregs are known to be especially potent downregulators of Th17 cell responses (Fletcher et al., 2009). Interestingly, all analysed regulatory immune cells were significantly increased in the synovial fluid compared to paired peripheral blood samples (Figure 25).

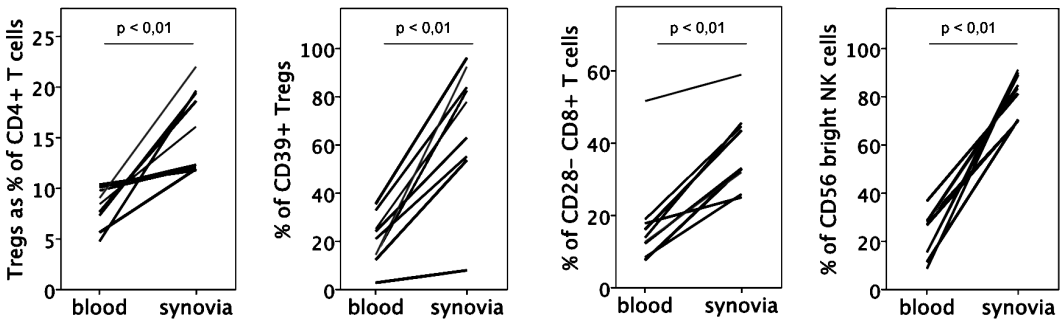

Figure 25 Frequency of regulatory immune cells in the peripheral blood and the synovial fluid. Shown in the figure is the frequency of a variety of immune cells with regulatory functions. Data was taken from the analysis of the "regulatory T cells", the "effector T cells" and the "subsets" antibody panel. Regulatory T cells are taken out of the CD4+ population, whereas CD39+ Tregs relate to the total amount of Tregs. Statistical significance was determined with a paired t-test.

The ectonucleases CD39 and CD73 play a role in the metabolims of the proinflammatory molecule ATP into adenosine, wherease CD26 can bind the adenosine desaminase that converts adenosine into inosine and hence eliminates an anti-inflammatory acting molecule. In the synovial fluid we found a significant increase of CD39 expressing conventional CD4+ and CD8+ lymphocytes. Unexpectedly, the expression of CD73 showed an opposite pattern to that of CD39 on T cells. As for CD26 we found a significant decrease in both CD4+ and CD8+ lymphocyte subsets in the synovial fluid. Interestingly, the expression of the late activation marker CD38 was diverging in CD4+ and CD8+ T cell. While the frequency of CD38+ CD4+ lymphocytes was significantly decreased in the synovial fluid we found an increase of CD38+ CD8+ T cells in inflammed joints.

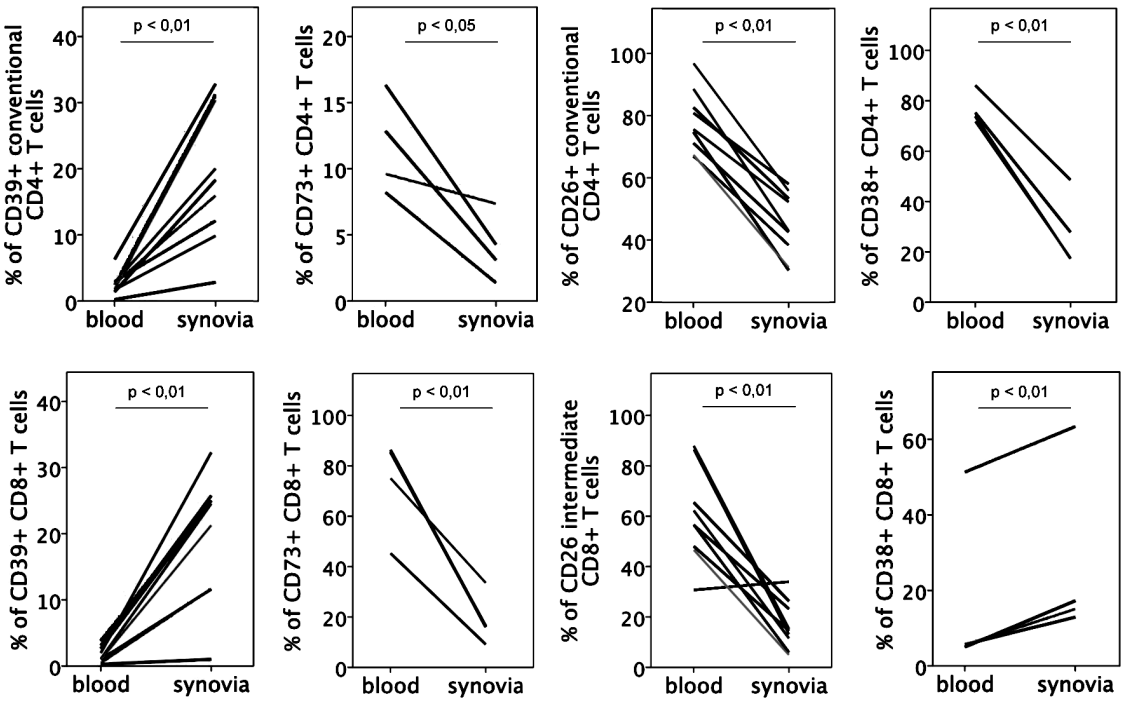

Figure 26 Frequency of CD4+ and CD8+ lymphocytes expressing the ectonucleases CD38, CD39, CD73 and CD26.
CD8+ T cells that highly expressed CD26 were excluded from this analysis because we identified them as a seperate cell subset, namely MAIT cells. A paired t-test was used to determine significant differences between the frequencies of subsets in peripheral blood and synovial fluid.

Innate-like lymphocytes: TCRγδ+ and MAIT cells in the synovial fluid

As shown in Figure 19, about half of the patients had an increase of TCRγδ+ T cells in the synovial fluid whereas the frequency of TCRγδ+ T cells was decreased in the rest of the patients. The function of TCRγδ+ T cell in JIA is not understood and different groups have reported data associating both inflammatory and regulatory features to TCRγδ+ T cell. Thus, we wanted to perform a detailed phenotypical analysis of surface markers, which are traditionally used in TCRαβ+ T cells, to see if through this we gain further insights into the role of TCRγδ+ T cells in JIA (Figure 27). In contrast to conventional TCRαβ+ T cells, the frequency of naïve TCRγδ+ T cells in the synovial fluid was higher compared to peripheral blood in most cases. Within memory cells, central memory (CD45RA- CD27+) TCRγδ+ T cells were predominant in the synovial fluid.

Analogous to TCRαβ+ T cells we found a dramatically increased frequency of TCRγδ+ T cells expressing the activation marker CD69 in the synovial fluid. In some patients up to 70% of the TCRγδ+ T cells showed an activated phenotype. In line

with this, there was also an accumulation of TCRγδ+ T cells lacking CD62L. IFNγ is the hallmark cytokine of TCRγδ+ T cells. Gamma delta T cells already showed large production of IFNγ in the peripheral blood. In contrast to TCRαβ+ T cells, the frequency of IFNγ-producing TCRγδ+ T cells was similar in the peripheral blood and the synovial fluid. Circulating human TCRγδ+ T cells from healthy donors did not produce IL-17. However, we observed that two of the patients showed IL-17 production in the peripheral blood. In the synovial fluid TCRγδ+ T cells of these patients even had a threefold increase of IL-17 production.

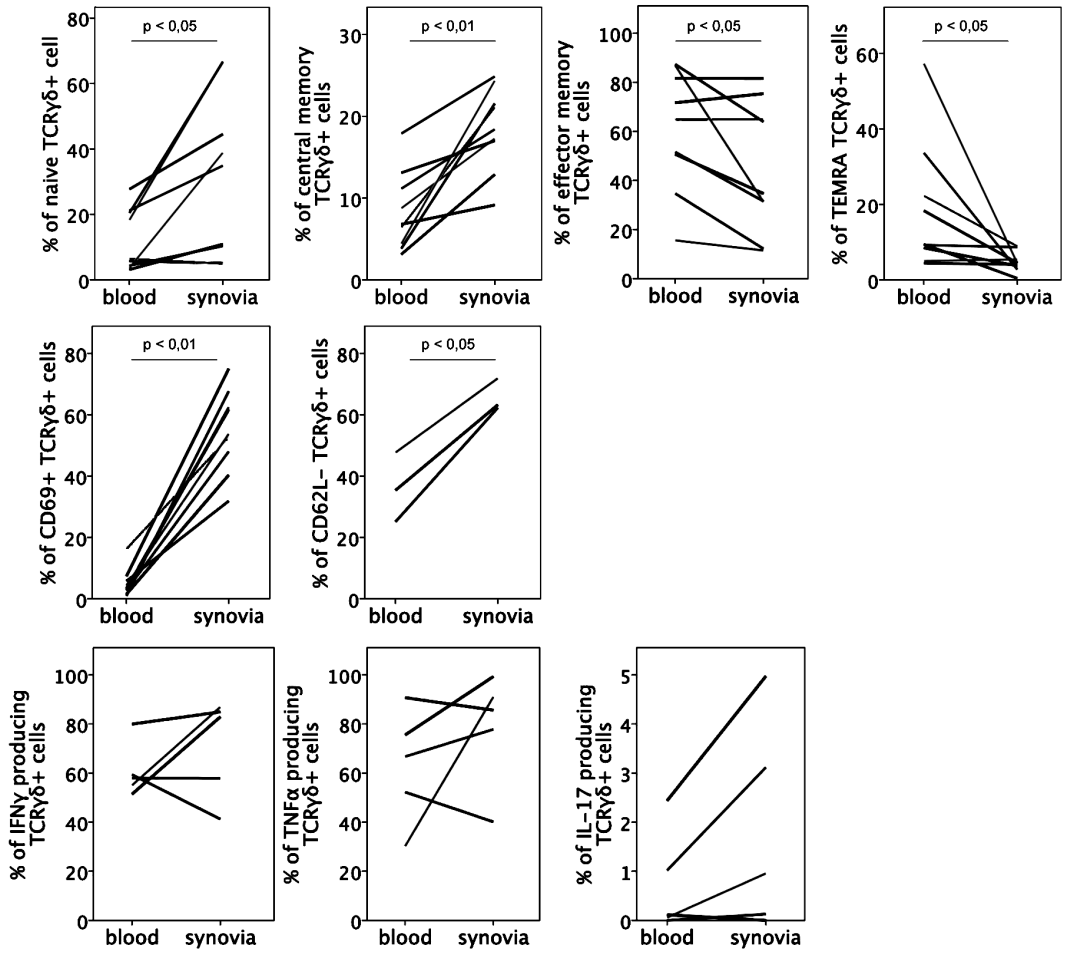

Figure 27 Phenotypical analysis of TCRγδ+ T cells in peripheral blood and synovial fluid.
The data for this figure was taken from the analysis of peripheral blood and synovial fluid that were stained with the antibody panels "effector T cells", "activated T cells" and "intracellular cytokine staining". The top panel shows the frequency of different maturational stages of TCRγδ+ T cells, the panel in the middle displays the frequency of activated TCRγδ+ T cells and in the bottom panel the frequency of cytokine producing TCRγδ+ T cells is shown. A paired t-test was performed to evaluate statistical significance.

Due to the downregulation of chemokine receptors in the synovial fluid we were more confident to mark MAIT cells as CD26^{high} CD8+ T cells, rather than as CCR6+ CD161+ CD8+ T cells in this compartment. In contrast to reports that MAIT cells are accumulated at the site of inflammation in MS (Illes et al., 2004), we found a significant decrease of CD8+ CD26^{high} T cells in JIA patients, although with our gating strategy we do not consider double negative MAIT cells, which typically represent about 10% of MAIT cells.

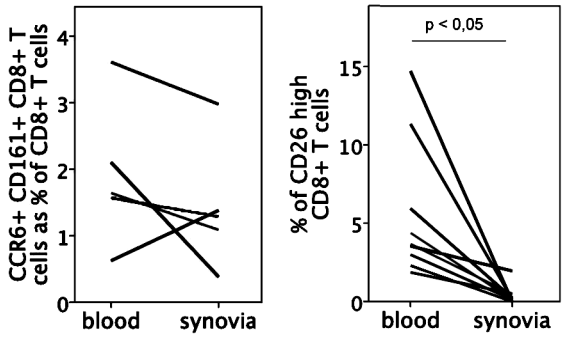

Figure 28 Frequency of MAIT in peripheral blood and synovial fluid.
The data for this figure is taken from the stainings of peripheral blood and synovial fluid cells with the "MAIT cells" and "regulatory T cells" panel. MAIT cells were identified as CD8+ CCR6+ CD161+ T cells or as CD8+ CD26high T cells. Statistical significance was determiend with a paired t-test.

In summary our data showed an accumulation of matured CD4+, CD8+ and B lymphocytes, increased frequencies of lympocytes expressing activation markers and a rise in all defined regulatory subsets in the synovial fluid of patients compared to peripheral blood. Additionally, IFNγ- and TNFα-producing lymphocytes were increased in the synovial fluid of all patients whereas the frequency of IL-17-producing CD4+ and CD8+ was only increased in half of the patients. Also in TCRγδ+ T cells we found an increased IL-17 production only in some patients whereas the production of TNFα and IFNγ was evenly high in peripheral blood and synovial fluid. In the synovial fluid we found an accumulation of naïve and central memory TCRγδ+ T cells and decreased amounts of effector TCRγδ+ T cells. Only a minority of TCRγδ+ T cells expressed the surface markers that define CD4+ and CD8+ TEMRA cells in the synovial fluid. CD39 expressing lymphocytes increased to different extends in the synovial fluid of all patients whereas we found a decreased frequency of CD73 and CD26 expressing CD4+ and CD8+ T cells at the site of inflammation.

Phenotypical analysis of peripheral blood of JIA patients and healthy donors

Mature TCRγδ+ T cells accumulate in the peripheral blood of untreated JIA patients

Synovial fluid samples are only available for a reduced set of patients. However, blood is drawn routinely in JIA patients giving us access to a much larger amount of samples. After finding that there were quite remarkable changes of leukocyte subsets in the peripheral blood and the synovial fluid of JIA patients we wanted to compare the leukocyte composition in peripheral blood of healthy donors and untreated JIA patients, independendly of disease entity. For the following analysis we used blood samples of 14 healthy donors and 34 JIA patients (10 oligoarthritis JIA, 18 polyarticular JIA, 6 enthesistis associated JIA). A Grubbs' test was performed within every population to determine outliers. After excluding outliers, we performed an unpaired t-test between healthy donors and JIA patients.

Most of the analysed subsets showed minimal alterations between healthy donors and untreated JIA patients (data not shown). However, we found striking differences in the TCRγδ+ T cell compartment. The frequency of TCRγδ+ T cells was significantly increased in the peripheral blood of JIA patients. Interestingly the frequencies of naive and central memory TCRγδ+ T cell were decreased in JIA patients whereas the effector memory TCRγδ+ T cell subset was increased. The only two other compartments showing significant differences were $CD56^{bright}$ NK cells and CD73+ CD4+ T cells. Both cell subsets were increased in the blood of untreated JIA patients (Figure 29).

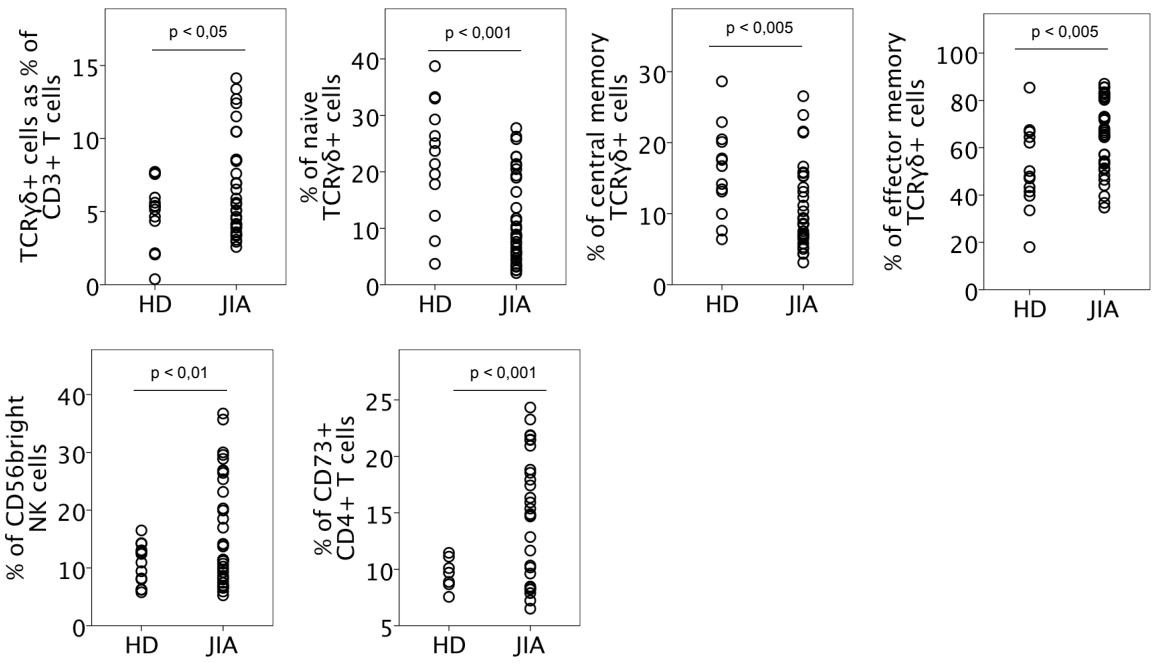

Figure 29 Comparison of leukocyte subsets in the peripheral blood of healthy donors and untreated JIA patients.
Shown in the figure are the frequencies of immune cells that significantly differ in healthy donors and untreated JIA patients. After identification of outliers, an unpaired t-test was performed to determine significant differences.

Phenotypical analysis of peripheral blood of JIA patients with different disease entities

All JIA subtypes show the same distribution of memory subsets in TCRγδ+ T cells

Since JIA is a very heterogenous disease, we next wanted to know if observed differences between healthy donors and untreated JIA patients were due to a single JIA entity or if the different subgroups of JIA showed similar distributions in inflammatory, regulatory, and innate like lymphocytes subsets. We established three groups of patients: 1. patients with oligoarthritis, 2. patients with polyarthritis (combining RF+ and RF- patients) and 3. patients with enthesitis-related JIA. We did not include patients with systemic arthritis and with psoriatric arthritis because of reduced sample size. After excluding outliers, we performed a multivariate analysis (ANOVA) comparing healthy donors with the different groups of patients.

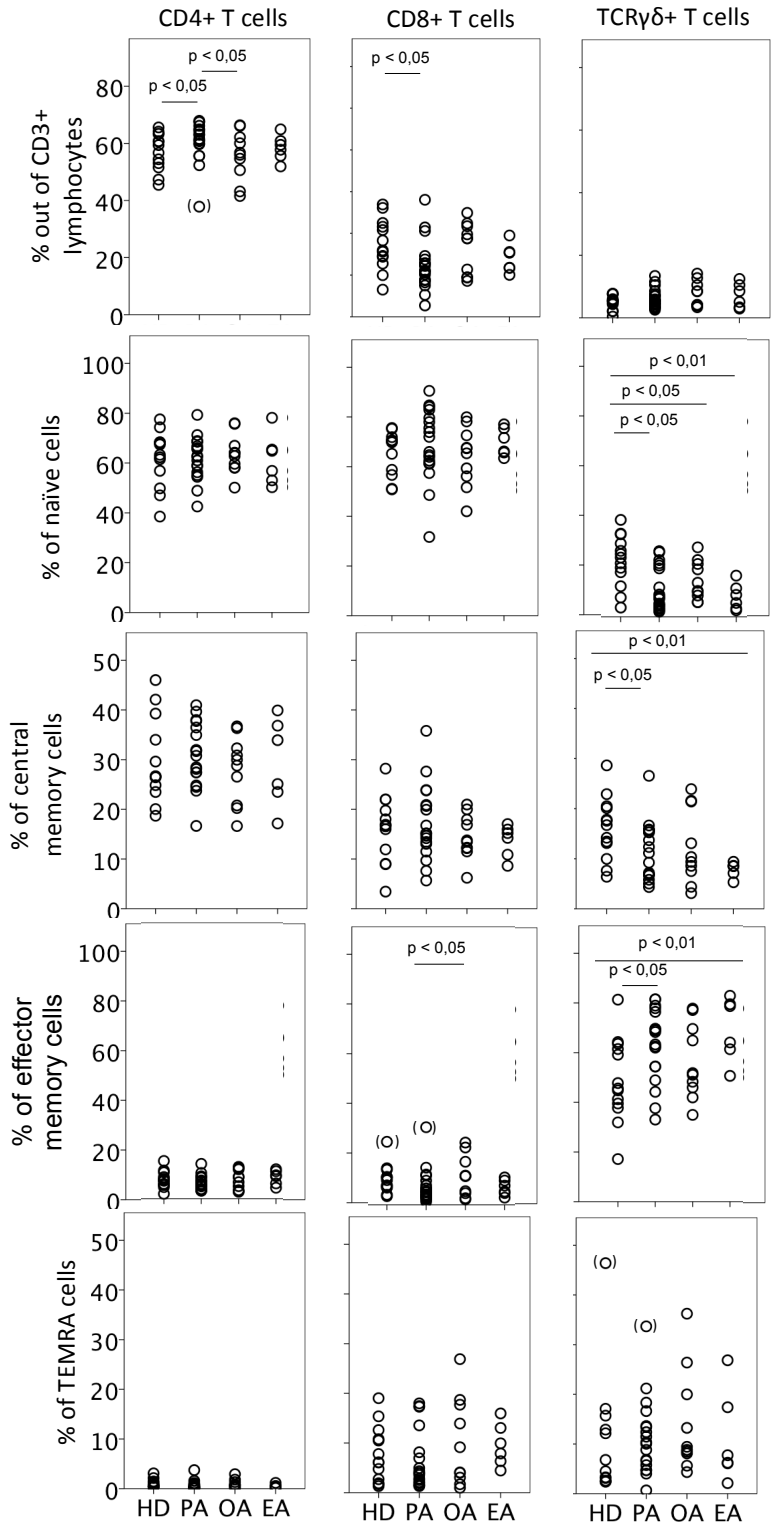

Figure 30 Comparison of memory subsets in TCRαβ+ and TCRγδ+ T cell subsets in the peripheral blood of healthy donors and untreated JIA patients of dfferent disease entities.
Populations are identified as described before. Outliers within each group are identified with the Grubb's test and are marked with (O) in the graph but are excluded from the statistical analysis. To determine differences we performed an ANOVA analysis.

Comparisons of healthy donors with the different JIA subgroups showed that the observed differences in described subsets were reflected in every disease subgroup. Although statistical significance was not reached we saw that independently from disease entity all untreated JIA patients had an increased frequency of TCRγδ+ T cells compared to healthy donors. Additionally, out data showed very clearly that the composition of memory subsets was similar in all JIA patients, namely a decrease in naïve and central memory TCRγδ+ T cells and an increase in effector memory TCRγδ+ T cells (Figure 30).

For TCRαβ+ T cells we saw that the distribution of naïve and memory cells were similar in healthy donors and all disease entities. Regarding cytokine production we also did not find many differences between healthy donors and untreated JIA patients of different disease subgroups. However, the frequency of TNFα-producing lymphocytes yielded some interesting aspects in children with enthesitis-related JIA. Untreated patients with enthesitis-related JIA had the least amount of TNFα production in both TCRαβ+ and TCRγδ+ lymphocytes compared to all other subgroups (Figure 31). Patients with oligoarthritis had a decreased frequency of IL-17-producing CD4+ T cells and the highest amount of CD69+ CD4+ T cells (data not shown). All other activation markers did not yield significant differences within the different disease groups.

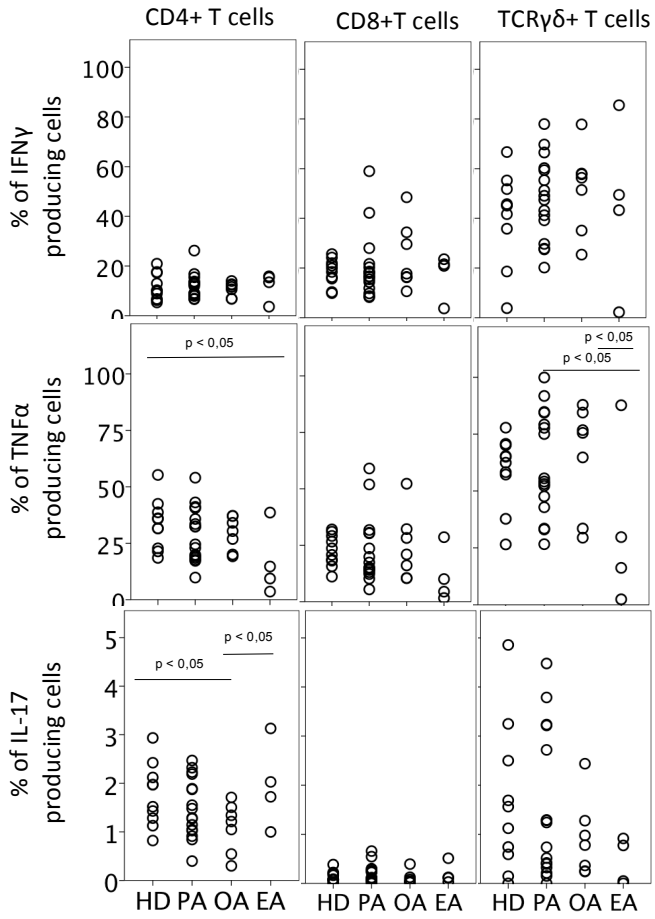

Figure 31 Cytokine production in CD4+, CD8+ and TCRγδ+ T cells in the peripheral blood of healthy donors and different JIA entities.

Within the regulatory subsets, patients with oligoarthritis had a significant increase in $CD56^{bright}$ NK cells and CD28- CD8+ T cells (data not shown). The frequency of all other regulatory subsets was similar in all disease subgroups. Within the ectoenzyme population we observed some differences among the patient cohorts. Our data showed that patients with oligoarthritis had the least amount of CD26+ CD4+ and $CD26^{intermediate}$ CD8+ T cells. Furthermore, it is remarkable that patients with enthesitis-related JIA had a decreased frequency of CD39+ CD4+ and CD8+ T cells. Of note, the increase of CD4+ CD73+ T cell that we had seen comparing untreated JIA patients with healthy donors was also reflected in every disease entity.

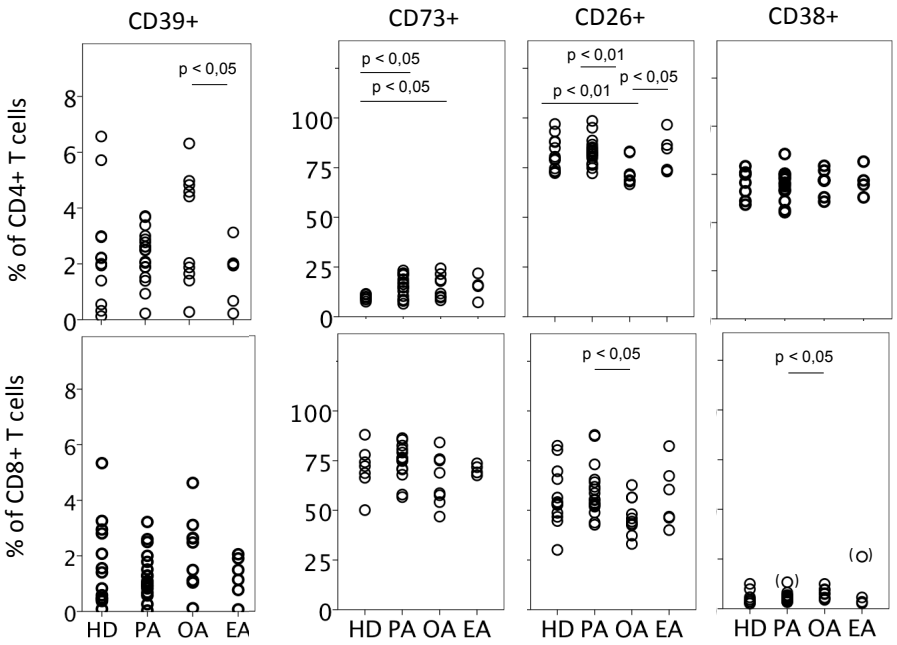

Figure 32 Frequency of CD4+ and CD8+ lymphocytes expressing the ectoenzymes CD39, CD73, CD26 and CD38.
Populations are identified as described before. After excluding outliers we performed an ANOVA analysis to determine significant differences.

In summary we could show that the differences we had found by comparing healthy donors with untreated JIA patients were also seen when comparing the three JIA entities separately. In our limited cohort, the comparison of all other leukocyte subsets within JIA subgroups did not yield many differences except that patients with enthesitis-related JIA had a reduced frequency of TNFα-producing cells and the least amount of CD39+ lymphocytes. In patients with oligoarthritis our data showed a reduced frequency of CD26+ lymphocytes and IL-17 producing CD4+ T cells, an increase of CD69+ CD4+ T cells and within the regulatory subsets an increased frequency of $CD56^{bright}$ NK cells and CD28- CD8+ T cells.

Patients treated with biologic agents have an increased frequency of inflammatory and regulatory leukocytes.

Finally, we were interested to which extend the patients' medication changes the leukocyte populations. For this analysis we included fourteen healthy donors, 35 untreated JIA patients, ten patients that received DMARDS and six patients that were administered with biologic agents. Patients that received biologics had a unique distribution of memory subsets in TCRαβ+ T cells. While the frequencies of naïve CD4+ and CD8+ lymphocytes were decreased in this patient cohort, we found a compensatory increase in both central memory and effector memory T cells. This arrangement of memory subsets reflects a more chronic inflammatory state in patients treated with biologics (Figure 33).

In TCRγδ+ T cells, the distribution of memory subsets was similar in all JIA patients but different to healthy donors. As we had already seen by comparing the different JIA disease entities, we also saw that in all treatment groups the frequency of naïve and CD45RA+ CD27+ central memory TCRγδ+ T cells was decreased while the frequency of CD45RA+ CD27- effector memory TCRγδ+ T cells is increased. The frequency of CD45RA+ CD27- TEMRA TCRαβ+ and TCRγδ+ T cells was similar in all analysed subgroups.

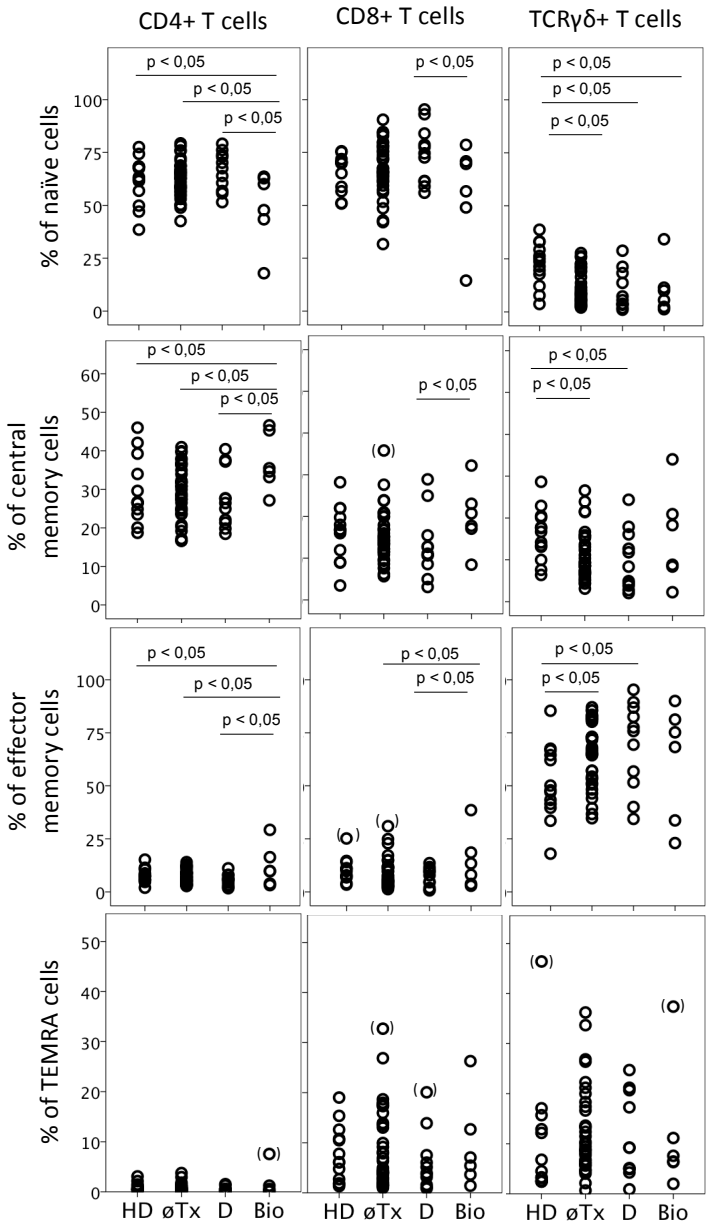

Figure 33 Frequency of memory subsets in healthy donors and JIA patients treated with different medications.
The Grubbs' test identified outliers which are marked as (O). To find out statistical significances an ANOVA analysis was performed comparing the frequencies of naive, central and effector memory and TEMRA lymphocytes in the peripheral blood of healthy donors, untreated JIA patients (øTx) and JIA patients treated with DMARDs (D) or biologics (Bio).

Increased inflammation in patients that were treated with biologics was also reflected by an increased frequency of CD69+ lymphocytes and an enhancement of CD4+ and CD8+ lymphocytes that produce IFNγ and TNFα. Interestingly in all treatment groups 1-4% of CD4+ lymphocytes produced IL-17. Of note patients receiving biologics had the least amount of IL-17-producing CD8+ T cells (Figure 34).

In the TCRγδ+ T cell compartment we also saw that patients that received biologics had the highest frequency of CD69+ cells. In contrast to the upregulation of this activation marker, we did not find an increased production of cytokines in this patient cohort. The frequency of IFNγ-producing TCRγδ+ T cells was similar in all subgroups. Of note, the frequency of IL-17-producing TCRγδ+ T cells was reduced in treated patients.

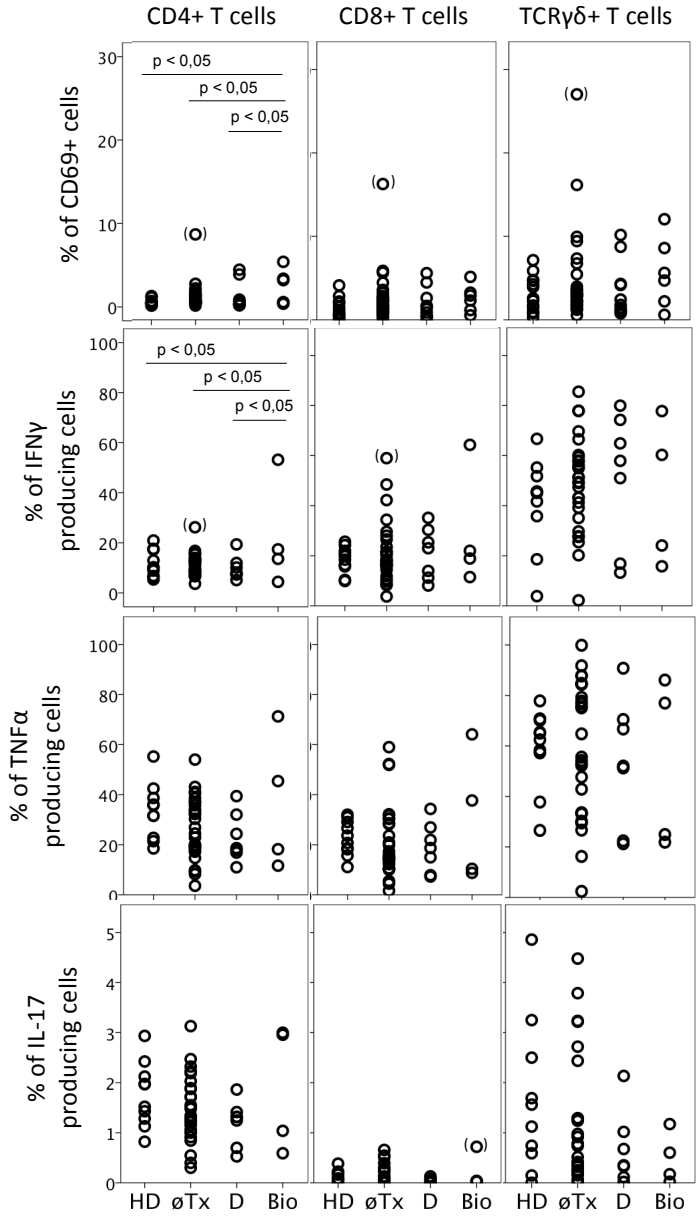

Figure 34 Frequency of activated and cytokine producing lymphocytes.
Shown in the figure is the frequency of CD69+ lymphocytes and the production of IFNγ, TNFα and IL-17 in TCRαβ+ and TCRγδ+ T cells. After excluding outliers (O) we performed an ANOVA analysis to identify significant differences between healthy donors, untreated JIA patients (øTx) and JIA patients treated with DMARDs (D) or biologics (Bio).

Although there was evidence of chronic inflammation in patients receiving biologics the frequency of regulatory T cells was similar in all groups. Nevertheless, CD39+ Tregs were significantly increased in patients treated with biologics. $CD56^{bright}$ NK cells that have been associated with regulatory functions were increased in untreated patients and in those treated with DMARDS while patients that received biologics had similar amounts like healthy donors (Figure 35).

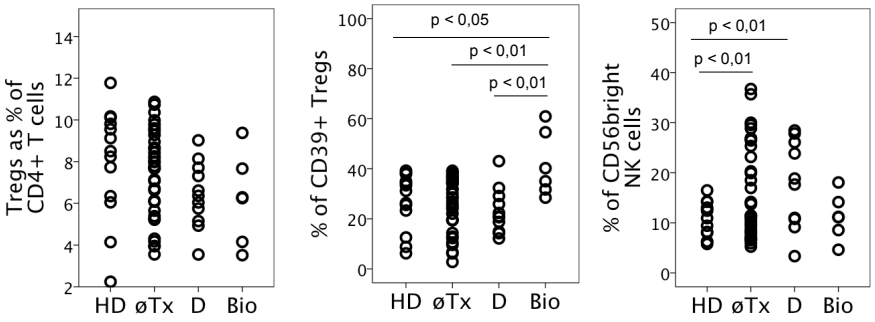

Figure 35 Frequency of regulatory subsets in peripheral blood of healthy donors and JIA patients treated with different medications.
After excluding outliers (O) we performed an ANOVA analysis to identify significant differences between healthy donors, untreated JIA patients (øTx) and JIA patients treated with DMARDs (D) or biologics (Bio).

In the CD4+ and CD8+ lymphocyte populations expressing ectoenzymes we found that the frequency of CD39+ T cells was similar in all groups. The expression of CD73 in CD4+ T cells was increased in all JIA patients and thus does not seem to be related to the effects of medication. Nevertheless, within CD8+ T cells we saw that patients treated with biologics had a decreased frequency of CD73+ CD8+ T cells.

The increased expression of CD26 in CD4+ and CD8+ lymphocytes in patients treated with biologics might be another indicator of the enhanced inflammatory processes. However, in contrast to all inflammatory markers analysed, the frequency of CD38+ lymphocytes was decreased in patients treated with biologics (Figure 36).

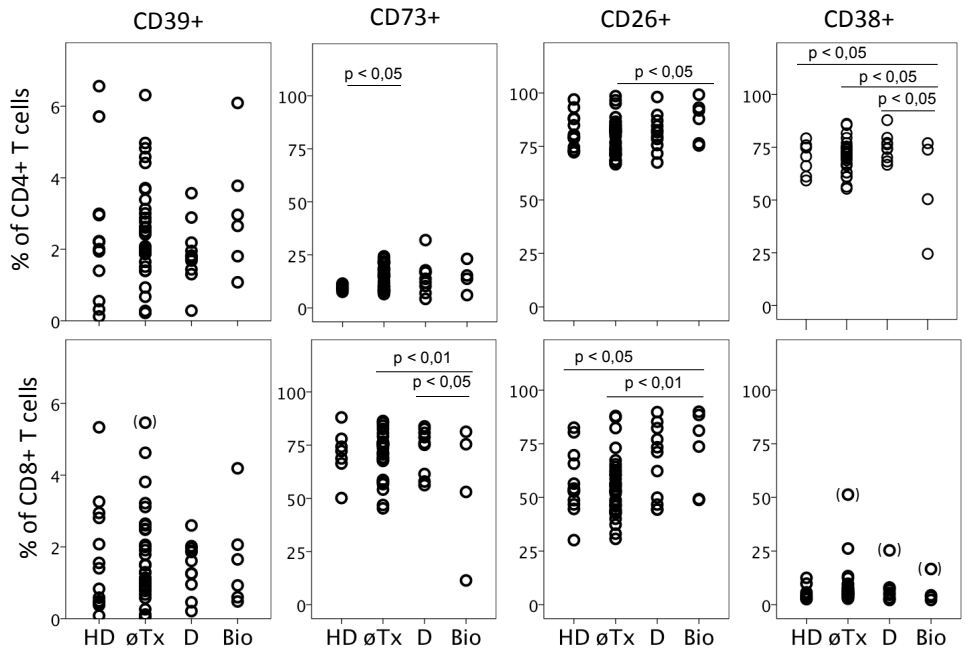

Figure 36 Frequency of lymphocytes expressing ectoenzymes in the peripheral blood of healthy donors and JIA patients receiving different medication.
After excluding outliers (O) we performed an ANOVA analysis to identfy significant differences between healthy donors, untreated JIA patients (øTx) and JIA patients treated with DMARDs (D) or biologics (Bio). The top panels shows the expression of ectoenzymes within CD4+ T cells, the lower panels shows the expression within CD8+ T cells.

In summary we found signs of increased inflammatory processes in patients receiving biologics. This was reflected by an increased frequency of CD69+, IFNγ and TNFα-producing TCRαβ+ T cells. Of note, we found a reduced production of IL-17, possbile due to an increase of CD39+ Tregs. The composition of the maturation status of CD4+ and CD8+ T cells in this patient cohort showed a shift to central and effector memory cell, indicating chronic inflammation. Concerning the expression of ectoenzymes we found a decreased frequency of CD73+ cells and in increase in the expression of CD26, both molecules involved in the adenosine metabolism.

DISCUSSION

JIA is a heterogenous group of childhood-onset arthritis consisting of various subtypes with distinct clinical and immunological features. Several groups have analysed the relevance of regulatory cell subsets (de Kleer et al., 2004) (Haufe et al., 2011), effector cell subsets (Nistala et al., 2010) (Bendersky et al., 2012) or cytokine production (Nistala et al., 2008) (de Jager et al., 2007) (Lin et al., 2011) in one or the other subtype of JIA. However, there has been no study so far integrating different immune cell types, T cell subsets, activation status of these cells, effector molecules produced, and even further, the innate like lymphocytes such as TCRγδ+ T cells and recently described MAIT cells. For this project we established a protocol that allowed the study of the phenotype of the above-mentioned cellular parameters in peripheral blood and synovial fluid of patients with JIA. Due to the heterogeneity of this condition, we stratified the different disease entities and treatment regimes. The aim of this study was to establish the existence of different "leukocyte profiles" in the peripheral blood or the synovial fluid of JIA patients, which might be used to predict the individual disease progression and enable a more adequate medical treatment for the patients.

Technical considerations for the phenotypic analysis

Multicolor flow cytometry is a powerful tool that allowed us to analyze several parameters simultaneously on a single cell basis. In this study we used eight-color flow cytometry to immunophenotype peripheral blood and synovial fluid of patients with JIA. For this, we developed eight antibody cocktails (Table 2) that enabled us to identify 62 different effector subsets. Our immunophenotyping analysis only required little amounts of material: 500µl of peripheral blood and 200.000 cells of synovial fluid. This is particularly important when analyzing samples of children since the amount of available material is very limited.

After the analysis of the first samples we had to adapt our panels. This was due to the identification of new subsets of interest and the availability of new fluorochrome labeled antibodies. Whenever an antibody panel was changed to integrate a new population we ensured that the former populations could still be

identified. The possibility of changing the panels offered the advantage to react to new subsets findings not only in JIA but also in other autoimmune diseases.

When combining the data from all patients and healthy donors to perform statistical analysis we encountered the problem that we had “missing data” whenever an antibody panel was not developed at time of sampling. In order to perform a multivariate analysis to identify typical “leukocyte profiles” that could predict disease progression, a complete data set of all subsets is required. Therefore, in this study we compared single markers in different groups to get a first insight into leukocyte subsets. We continued to collect more samples of JIA patients and healthy donors to be able to perform a reasonable multivariate analysis. Missing data from patients that have already been included in the study could be filled repeating specific stainings with frozen material, which has been shown to yield same results as fresh material in terms of phenotyping (Weinberg et al., 2009).

Cell subsets were usually identified by the expression of one unique combination of surface or intracellular markers. However, for Th1, Th17 and MAIT cells we used two identification strategies with the goal to find the easiest and most practicable way of phenotyping. First, we identified Th1 and Th17 cells as CD4+ lymphocytes that produce IFNγ and IL-17, respectively. Second, we used a combination of chemokine receptors. Intracellular cytokine staining requires a minimum of 100.000 cells, *in vitro* stimulation and a large logistic effort. Since we only had access to very small amounts of samples it was desirable to assess the main cytokine producing cells with a simple surface staining without additional stimulation and fixation steps. It has been previously shown that CCR4 and CXCR3 are useful markers to identify IFNγ-producing Th1 cells, (reviewed by (Sallusto and Lanzavecchia, 2009), whereas IL-17-producing cells can be identified with the markers CCR4 and CCR6 (Acosta-Rodriguez et al., 2007). In peripheral blood we could show that both strategies to identify Th1 and Th17 were successful, whereas in the synovial fluid we did not find a clear correlation. This could be due to the sample size or to the fact that the pattern of expression of chemokine receptors changes when T cells enter the target site (Chen et al., 2004) (Cosmi et al., 2008). Therefore, in an inflammatory compartment like the synovial fluid of JIA patients, we suggest to use intracellular cytokine staining to identify Th1 and Th17 cells.

Recently identified MAIT cells belong to the compartment of innate like lymphocytes and are commonly identified by the expression of the invariant T cell

receptor chain Vα7.2 (Martin, Treiner et al. 2009) and by high expression of CD161. The majority of MAIT cells are CD8+, less than 10% are double negative and very few MAIT cells express CD4 (Walker et al., 2012). Concerning cytokine production, about 10% of MAIT cells secrete IL-17 and 25% produce IFNγ (Dusseaux et al., 2011). Because of their capacity to produce IL-17, they are an interesting cell subset in the context of inflammation and autoimmunity. Indeed, MAIT cells have been found in the target sites of MS (Miyazaki et al., 2011) and in collagen-induced arthritis, the animal model for arthritis (Chiba et al., 2012). At the start of this study, the antibody against Va7.2 was not commercially available. However, based on the expression profile of MAIT cells reported by Dusseaux (Dusseaux et al., 2011), we used two strategies to analyze CD8+ MAIT cells: first as $CD26^{high}$ CD8+ cells and secondly as CCR6+ CD161+ CD8+ cells. We could show that the frequencies of MAIT cells identified with both strategies clearly correlated with each other. This is an important finding because we developed the “MAIT cells” antibody panel with the antibodies against CCR6 and CD161 at a later time point and, therefore, did not have this data of all patients. By showing that both identification strategies serve to analyse CD8+ MAIT cells, we could identify these cells in all samples since the antibody against CD26 was part of our “regulatory T cell antibody panel” from the beginning of the study.

Phenotypical analysis of immune cells in the blood of healthy donors and JIA patients

Target organs of autoimmune disease are filled with inflammatory cells. However, access to the target organs is challenging in many autoimmune diseases, such as diabetes mellitus and MS. Immunological studies in these diseases are usually restricted to the analysis of the peripheral blood, which is available from a large cohort of patients, but it does not always reflect the changes found in the target organs.

There have been various attempts to find biomarkers in peripheral blood that mirror the manifestation of an autoimmune disease process. In systemic lupus erythematosus (SLE) a dysregulated expression of genes belonging to the type 1 IFN pathway has been found in PBMCs of patients with a severe course of disease (Baechler et al., 2003). Thus, immune profiling in SLE may help to find those patients

with a more aggressive disease progression and may identify patients that benefit from a therapy specifically targeting impaired immune response mechanism. In MS, extensive immunophenotyping using flow cytometry has shown that the only peripheral blood subset that differs between untreated patients with relapsing-remitting MS and healthy donors are $CD8^{low}$ CD4- lymphocytes. This lower frequency of $CD8^{low}$ CD4- lymphocytes is useful to discriminate disease progression but is not a diagnostic marker for MS (De Jager et al., 2008). In systemic JIA studies aimed to predict a higher risk to develop a severe disease course have yielded a cluster of genes related to development of the macrophage-activating syndrome (Fall et al., 2007). In another study, flow cytometry was used to analyze 13 different lymphocyte subsets regarding lineage, activation and differentiation in the peripheral blood of patients with different JIA subtypes. The group found that patients with oligo- and polyarthritis have an increased amount of activated HLA-DR+ CD4+ and CD8+ T cells and CD19+ B cells. In contrast, patients with systemic JIA had no abnormalities in the composition of T cells, but showed decreased amounts of NK cells, especially those expressing CD57 (Wouters et al., 2002). However, this study did not find good indicators to evaluate the progression of disease in individual patients.

Comparing our 62 defined leukocyte populations in the peripheral blood of healthy donors and untreated patients the only significant difference that we found occurred in the frequency of TCRγδ+ T cells, $CD56^{bright}$ NK cells and CD73+ CD4+ T cells. Conflicting data has been published about the role of TCRγδ+ T cells in JIA. Some studies point out inflammatory features, since the majority of TCRγδ+ T cells in the peripheral blood and the synovial fluid of JIA patients produce proinflammatory cytokines such as IFNγ, TNFα and IL-17 (Bendersky et al., 2012). In contrast, a different study claims that TCRγδ+ T cells are rather associated with regulatory functions, because high amounts of TCRγδ+ T cells in the synovial fluid of patients correlate with a less severe disease progression (Berkun et al., 2011). Our data showed that JIA patients had a significantly increased frequency of TCRγδ+ T cells in peripheral blood compared to healthy donors. This is in contrast to previously published data, which described no significant changes in the frequency of TCRγδ+ T cells in the peripheral blood of JIA patients and healthy donors (Wouters et al., 2002). However, this study included a very limited amount of patients. Within the TCRγδ+ T cell population of JIA patients, we found a decrease in naïve and central memory and an increase in effector memory TCRγδ+ T cells. Interestingly, we did not observe this

trend in CD4+ T cells and only very slightly in CD8+ T cells, indicating that TCRγδ+ T cells may play an important role in the pathogenesis of JIA. A unique property of TCRγδ+ T cells is that they are able to present antigen to other T cells possibly driving synovitis in JIA: Upon activation, peripheral blood Vδ2+ TCRγδ+ T cells express HLA-DR, CD80 and CD86 (Brandes et al., 2005) which are molecules that mark antigen-presenting cells. Additionally, TCRγδ+ T cells secrete proinflammatory cytokines such as IFNγ and IL-17 (De Rosa et al., 2004, Moens et al., 2011) (Kenna et al., 2012). Since they are readily activated also independently of the T cell receptor (Sutton et al., 2009) (Martin et al., 2009a), they can induce fast local inflammatory processes, which may contribute to synovitis. Thus, effector memory TCRγδ+ T cells may contribute to the immune dysfunction and may promote and sustain inflammatory processes in inflamed joints of patients with different arthritis disease groups.

After synovial inflammation is controlled, effector memory TCRγδ+ T cells may re-enter the blood stream. This could explain our observed differences in the frequencies of naïve and memory TCRγδ+ T cells between JIA patients and healthy donors. The question arises why the same pattern is not found in CD4+ T cells. One explanation could be that CD4+ T cells have a higher diversity of the T cell receptor and play an important role in the immune defense of a large set of infectious agents in children. Because of the huge diversity of the CD4+ T cell receptor, the amount of CD4+ memory cells arising from synovial inflammation is relatively small compared to the total number of CD4+ memory T cells in peripheral blood. This could explain why we could not detect differences in the memory populations of CD4+ T cells in the peripheral blood of JIA patients compared to healthy donors.

Comparison of immune cell subsets in different JIA entities

Based on the clinical presentation, several laboratory findings and the patients' history, JIA is classified into seven subgroups: systemic JIA, oligoarthritis, RF+ and RF- polyarthritis, enthesitis-related JIA, psoriatic JIA and undifferentiated JIA (Petty et al., 2004). Oligo- and polyarthritis are classical T cell driven autoimmune diseases involving immune defects of the adaptive immune system, leading to local joint inflammation (Lin et al., 2011). In contrast to this, the systemic form of JIA is considered an autoinflammatory disease presenting with dysfunctions of the innate

immune system resulting in symptoms as fever, skin rash, hepato-splenomegaly and joint pain (Pascual et al., 2005, de Jager et al., 2007). Both enthesitis-related JIA and psoriasis JIA are secondary to other autoimmune diseases: Enthesitis-related JIA is linked to ankylosing spondylitis and inflammatory bowel disease (Chen et al., 2004), while patients with psoriasis JIA present with skin lesions typical for psoriasis or have family relatives with psoriasis (Petty et al., 2004). Since JIA is a very heterogeneous disease, we wanted to compare immune subsets within different disease subgroups.

Our patients collective consisted of only few patients with systemic JIA and psoriasis JIA, so that we focused our analysis on the comparison of immune subsets of patients with oligo-, polyarthritis and enthesitis-related JIA. Independently of the disease entity, all JIA patients showed an increase of the frequency of TCRγδ+ T cells and an increase of effector memory TCRγδ+ T cells in the peripheral blood compared to healthy donors. Thus, the composition of memory subsets in TCRγδ+ T cells is a general trend in all analysed JIA subgroups. Furthermore, our data showed that the frequencies of all other leukocyte subsets were similar in all disease entities: In the TCRαβ+ T cells compartment we found similar levels of activation and comparable frequencies of memory and naive cells. Within the regulatory subsets we saw that patients with oligoarthritis had an increase of $CD56^{bright}$ NK cells. The frequency of all other regulatory subsets was similar in all disease groups. Comparing cytokine production we observed that the frequency of IFNγ-producing CD4+ and CD8+ lymphocytes was similar in healthy donors and all JIA subgroups. Although not reaching significance our data showed that the frequency of IFNγ-producing TCRγδ+ T cells was increased in all disease subgroups possibly indicating an augmented activation status.

In contrast, the frequency of IL-17- and TNFα-producing lymphocytes revealed alterations in different JIA disease subgroups. IL-17 is a cytokine associated with autoimmune inflammation (Brucklacher-Waldert et al., 2009, Annunziato et al., 2008, Awasthi and Kuchroo, 2009). In JIA, IL- 17 has pleiotropic effects and is associated with damage of cartilage and bone (Agarwal et al., 2008). In our study, we found the highest frequency of IL-17-producing CD4+ T cells in the peripheral blood of healthy donors and patients with enthesitis-related JIA. The frequency of IL-17-producing CD4+ T cells of patients with oligo- and polyarthritis was decreased compared to healthy donors, possibly because IL-17-producing precursors preferentially migrate to the inflamed joints. In the peripheral blood of healthy donors about 2% of TCRγδ+ T

cells produced IL-17 while in JIA patients the frequency was slightly reduced. This is in conflict with a different study, which found that 8% of Vy9 and 30% of Vδ1 T cells produced IL-17 in JIA patients (Bendersky et al., 2012). However, in this study only patients with an acute flair were analyzed. Thus, this patient cohort was rather different from ours.

Another cytokine found in inflamed joints is TNFα. TNFα has a broad spectrum of proinflammatory properties, activating macrophages and leading to an increased production of inflammatory cytokines. Both in rheumatoid arthritis and JIA, blockage of TNFα has turned into a very successful therapy approach (Prince et al., 2009, Quartier et al., 2003). Interestngly, in patients with enthesitis-related JIA the blockage of TNFα is less sucessful (Donnithorne et al., 2011).

Regarding TNFα production our data showed that patients with enthesitis-related JIA had the lowest amount of TNFα-producing CD4+, CD8+ and TCRγδ+ T cells compared to all other groups. However, we did not address TNFα production by monocytes.

There are a few studies indicating that the pathogenesis of enthesitis-related JIA differs from poly- and oligoarthritis. While in patients with oligo- and polyarthritis it is mainly autoreactive T cells that seem to trigger synovial inflammation, there is evidence that the innate immune system also plays a prominent role in the enthesitis-related form of JIA, where autoantibodies are rare. (Saxena et al., 2006) (Myles and Aggarwal, 2011) (Myles et al., 2012). In the adult form of spondyloarthritis, which shares characteristics with enthesitis-related JIA, the lack of association with typical autoimmune genes, absence of autoantibodies and the only modest response to T and B cell targeted therapies lead to the hypothesis that the adaptive immune system is not primarily of importance in this disease group (Ambarus et al., 2012).

In contrast to the studies indicating major pathogenic influence of the innate immune system, the association of the MHC class I molecule HLA-B27 in patients with enthesitis-related JIA has served as an indicator of a dominant role of the adaptive immune system in these patients. HLA-B27 can present bacterial peptides and consequently activates cytotoxic T cells, which then cross react with self peptides in the joints (Hermann et al., 1993). However, this “arthritogenic peptide concept” was challenged by recent studies: The first study observed that homodimers of HLA-B27 by itself can induce NK and T cell activation (Kollnberger and Bowness, 2009). Upon activation NK and T cells produce increased amounts of

proinflammatory mediators, which could lead to synovitis. Another study arguing against the “arthritogenic peptide concept” claims that a misfold of HLA-B27 in the endosplamatic reticulum leads to an NF-kB dependent inflammatory response, resulting in Th17 cell activation (Colbert et al., 2010). Thus, the association with HLA-B27 is not in conflict with the assumption that the pathogenesis of enthesitis-related JIA involves both innate and adaptive immune responses.

An obvious limitation of our study is the small sample size consisting of 52 patients and 14 control patients. When separating the JIA samples into different disease entities the sample size per groups even decreases more, challenging reasonable statistical analysis. Finding adequate control samples especially of very young children is also difficult since blood of healthy children is not easily accessible. Most children are seen in the hospital with infectious diseases and, therefore, do not serve as good controls.

In agreement with other studies (Wouters et al., 2002) (Nistala et al., 2008) we find that there were only minimal changes in the leukocyte composition in the peripheral blood of JIA patients. However, patients with enthesitis-related JIA showed a reduced frequency of TNFα-producing TCRαβ+ and TCRγδ+ T cells, possibly pointing to a pathogenic role of the innate immune system. The peripheral blood of patients with oligo- and polyarthritis did not yield many differences in the composition of immune cells. Further immunophenotyping studies including more patients, immune subsets and effector molecules, e.g. monocytes, dendritic cells, IL-6 and IL-10, have to be conducted to get a more detailed insight into possible differences in JIA subtypes. Additionally, immunophenotyping of synovial fluid samples of patients with enthesitis-related JIA compared to patients with oligo- and polyarthritis would help to analyse possible differences in the pathology of these JIA subgroups. Unfortunately we did not have access to synovial fluid from this patient cohort and, therefore, cannot state if our observed differences in cytokine profile of peripheral blood T cells are also found at the site of inflammation.

New thoughts on the classification of JIA subgroups

One of the reasons why we did not find many differences in the peripheral blood of different JIA subgroups may also be the current classification in use. The similarities of the leukocyte composition in patients with oligo- and polyarthritis raises the

question if these two disease entities are really unique subgroups. Clinically they differ in the number of affected joints. From an immunological point of view we found similar features in the activation status of lymphocytes, the composition of memory cells and the frequency of regulatory cells. A study using multiplex immunoassays for the analysis the cytokine profile in different JIA subgroups and rheumatoid arthritis, the plasma and synovial fluid of oligo- and polyarticular JIA patients showed similar biomarker profiles (van den Ham et al., 2009).

Because of the similarities in oligo- and polyarthritis and the heterogeneity in other JIA subgroups, there is a raising amount of authors demanding a new classification of JIA including more biologic markers (reviewed by (Martini, 2012). Systemic JIA has already been classified as a unique subset of JIA, because of its features as an autoinflammatory disease, involving an activation of the innate immune system. This is underlined by the poor response to TNFα and the successful treatment with IL-1 and IL-6 (Quartier et al., 2003) (Herlin, 2010). Concerning other JIA subgroups, patients with positive ANAs share similarities in the presentation and progression of disease in contrast to patients with no ANA antibodies (Ravelli et al., 2005) (Ravelli et al., 2011). However, in the current classification of JIA, ANA+ patients are found in several subgroups: in patients with oligoarthritis, RF+ and RF- polyarthritis, and with psoriasis JIA. Also the group of patients with psoriasis arthritis does not form a heterogeneous patient collective but rather two major subgroups: one that clinically presents with features of the enthesitis-related form and the other sharing similarities with ANA+ oligoarticular patients (Stoll et al., 2006). Martini et al suggest that features indicating a common cause of disease, such as sex ratio, age of onset, asymmetry of arthritis and ANA positivity should be used for classification (Martini, 2003). Imagining techniques such as ultrasonography and MRI scan assessing synovitis could also be a useful tool to classify JIA subgroups (Magni-Manzoni et al., 2009). Especially the differentiation between tendon and articular inflammation could be made more adequately (Rooney et al., 2009). Thus, the differentiation between enthesitis-related JIA, which is typically associated with inflammation at the insertion of tendons, and other entities of JIA would be more objective.

The joints of JIA display signs of inflammation

Children are usually punctured during flares of disease to relieve the joint of the inflammatory synovial fluid in order to prevent further joint damage and disability. Analysis of the composition of lymphocytes in paired samples of peripheral blood and the synovial fluid revealed an accumulation of CD8+ T cells. This finding fits to prior studies that also found a predominance of CD8+ T cell in the synovial fluid of patients with rheumatoid arthritis and JIA (Murray et al., 1996) (Haworth et al., 2008) (Hunter et al., 2010). One study has shown that patients with oligoarthritis are more likely to develop a more severe course of disease if they have a low CD4:CD8 ratio in the synovial fluid. Unlike in diabetes mellitus type 1, where CD8+ T cells contribute to the pathogenic lysis of pancreatic cells (Roep, 2008), no clear pathological features of CD8+ T cells have been found in JIA. The ratio of CD4 and CD8 T cells may rather reflect a chemokine milieu that favors the migration of CD8+ T cells. CCL5 is an example of a chemokine that is increased during synovitis (Pharoah et al., 2006) and preferentially attracts CD8+ T cells (Hunter et al., 2010).

Compared to peripheral blood, the synovial fluid contained more mature activated cells, but also regulatory leukocytes. In line with other studies (Gattorno et al., 2005), we found an increase of effector memory cells in the synovial fluid. Increased frequencies of CD4+ and CD8+ effector memory T cells have also been found in the synovial fluid of patients with rheumatoid arthritis (Ezawa et al., 1997, Cho et al., 2012).

The frequency of B cells was lower in the synovial fluid compared to peripheral blood of JIA patients, but the few cells that we found in the synovial fluid all belonged to the memory subset, as it has been already described (Corcione et al., 2009). Remarkably, plasma cells were increased. It has been shown that B cells in the inflamed joints express surface markers typical for antigen presenting cells and thus may have an antibody independent role in chronic inflammation (Morbach et al., 2011). Summarising our findings, the shift to mature T and B lymphocytes in the joints of JIA patient reflects the ongoing inflammatory processes, also found in rheumatoid arthritis.

As further indicators of ongoing inflammation, the activation markers CD25 and CD69 were upregulated in all synovial fluid lymphocytes. Additionally, there was a higher frequency of T cells that have shed CD62L, which occurs upon activation.

The upregulation of CD69, CD25, CD71 and HLA-DR on synovial fluid T cells has been described in the context of JIA (Black et al., 2002). As other groups have reported, we also found a significant increase in IFNγ and TNFα producing CD4+ and CD8+ T cells contributing to the inflammatory milieu in the joint (Wedderburn et al., 2000). Our data shows that IL-17-producing cells were increased in some patients, but global significance was not reached. One patient had a striking amount of 30% IL-17-producing CD4+ T cells and 10% CD8+ T cells. There are several studies that state that the inflamed joints of JIA patients are enriched with IL-17-producing T cells (Nistala et al., 2008) (Agarwal et al., 2008). Nevertheless, in the study of Nistala only about half of the patients had a clear increase of Th17 cells in the synovial fluid compared to peripheral blood whereas the other had similar amounts. Higher frequencies of Th17 cells were seen in patients presenting with a worse clinical phenotype and with fewer regulatory cells in the synovial fluid (Nistala et al., 2008, Olivito et al., 2009). This indicates a stronger imbalance between inflammatory and regulatory subsets in patients with a more severe progression of disease

It can be speculated if patients that have such an increased production of IL-17-producing T cells require a specific treatment that neutralizes IL-17 or the IL-23/IL-17 axis. Cytokine profiling of the inflamed joints might be a good predictor for the need of a more or less aggressive therapy plan or a specific cytokine therapy. Patients with an increased production of cytokines may profit from an early introduction of biologics specifically targeting cytokines that are prominent in an individual.

In agreement with other groups (Hunter et al., 2010) (Dalbeth and Callan, 2002), we found a dramatic increase in the frequency of $CD56^{bright}$ NK cells in the synovial fluid. $CD56^{bright}$ NK cells are associated with regulatory features and are less cytotoxic than their $CD56^{dim}$ counterparts and rather produce cytokines. Functional analysis indicates that $CD56^{bright}$ NK cells express activation markers and are a source of IFNγ (Dalbeth et al., 2004). IFNγ can function in an immune regulatory way driving CD4+ differentiation to the Th1 subtype and away from the Th17 phenotype. Additionally, IFNγ inhibits osteoclast differentiation and subsequently bone destruction. On the other hand, it was also shown that $CD56^{bright}$ NK cell promote TNFα production by monocytes and thereby amplify the inflammatory response in the joints (Dalbeth et al., 2004).

Regulatory subsets accumulate in the inflamed joints

Inflammation is usually controlled by regulatory cell subsets. However, in autoimmune disease, the function of regulatory T cells is often impaired (Viglietta et al., 2004). Our data showed that all regulatory subsets, namely Tregs, and especially the highly suppressive subset CD39+ Tregs and $CD56^{bright}$ NK cells were significantly increased in the joints of JIA patients compared to peripheral blood.

In agreement with published data (de Kleer et al., 2004), we have seen that the frequency of Tregs was greatly increased in the synovial fluid. Previously it has been shown that patients with persistent oligoarthritis have higher expression levels of FOXP3+ Tregs compared to patients with the more severe form of extended oligoarthritis, indicating that the level of FOXP3 correlates with the clinical subtype and course of disease (de Kleer et al., 2004). The same group reported an inverse relationship between IL-17-producing T cells and Tregs, confirming that patients with a more severe subtype of JIA have a lower frequency of regulatory T cells and increased amounts of IL-17 in the synovia (Nistala et al., 2008).

We, as others (Moncrieffe et al., 2010), could further show that within Tregs the subset of CD39+ Tregs accumulate at the site of inflammation. CD39+ Tregs contribute to the degradation of the rather proinflammatory ATP to generate adenosine, which has several anti-inflammatory effects. Additionally CD39+ Tregs are particularly efficient at reducing cytokine production by effector T cells (Fletcher et al., 2009) (Rissiek et al., 2015). It is still unclear, if CD39+ Tregs might selectively migrate to the site of inflammation, or if CD39 is upregulated within the joint to control inflammatory processes.

Since regulatory subsets are elevated at the site of inflammation, it is unclear why mechanisms to dampen inflammatory processes in the joints fail. A reason could be that they still lack in quantity, or that their ability to control inflammation is impaired, as shown in MS (Fletcher et al., 2009) (Viglietta et al., 2004). Alternatively, it could be that effector cells are more resistant to suppression (van Amelsfort et al., 2007) (Valencia et al., 2006), since regulatory T cells isolated from JIA patients are not functionally impaired *in-vitro* (Moncrieffe et al., 2010) (Bendersky et al., 2012) and are capable of suppressing conventional T cells from peripheral blood of healthy donors (Haufe et al., 2011). In agreement with this concept, CD69+ HLA-DR+ effector T cells isolated from inflamed joints of JIA patients have a decreased

susceptibility to regulatory T cells from both healthy donors and JIA patients (Haufe, Schepp et al. 2011). Our data showed a dramatic increase in these CD69+ T cells in the joint which may be the reason for the impairment of immune regulation.

Ectoenzymes as new target option in JIA therapy

ATP and adenosine contribute to the configuration of the inflammatory milieu within the joint. ATP is a proinflammatory molecule that stimulates inflammatory cytokine production and induces the generation of Th17 cells (Atarashi et al., 2008), while adenosine is associated with antiinflammatory functions, namely the inhibition of T cell proliferation and inflammatory cytokine production (Zarek et al., 2008). CD39 hydrolyses ATP into AMP (Dwyer et al., 2007), which may reflect a mechanism to dampen inflammatory responses of ATP and to restore immune balance. We, as others (Moncrieffe, Nistala et al. 2010), also observed increased amounts of CD39 on CD4+ conventional T cells and on CD8+ T cells in the synovial fluid, probably indicating a more severe inflammatory state. Of note, the increase in the frequency of CD39+ lymphocytes varied substantially among the patients. It has been recently shown that CD39 expression on Tregs is genetically determined and can be moderately altered through activation (Rissiek et al., 2015).

AMP is further metabolized into adenosine by the ectonuclease CD73. Extracellular adenosine binds to the A2A receptor of T cells and induces an intracellular cascade resulting in decreased production of IL-2 and IFNγ of effector T cells (Zarek et al., 2008). Blocking of CD39 or CD73 inhibits the immune regulating effect of adenosine (Mandapathil et al., 2010). In line with previous data, we saw a decreased frequency of CD73+ CD4+ T cells, indicating an insufficient breakdown of the proinflammatory ATP into the immune regulatory adenosine in the synovial fluid of JIA patients (Moncrieffe, Nistala et al. 2010) (Botta Gordon-Smith et al., 2014). A recently published study shows that lymphocytes of the synovial fluid show a decreased AMPase activity compared to peripheal blood (Botta Gordon-Smith et al., 2014). The accumulation of AMP and the lack of adenosine could also contribute to a local cytokine milieu, which leads to the hyporesponsiveness of effector T cells challenging immune regulation.

In the mouse model of CIA, A2A receptor agonists reduce inflammation in arthritic joints of mice (Flogel et al., 2012). Finding a way to upregulate CD73 or to

increase the amount of adenosine in the inflamed tissue may be a promising approach in JIA therapy. So far there have been several studies discussing that methotrexate, among other mechanisms of action, promotes the release of adenosine (Cronstein et al., 1991) (Cronstein et al., 1993) in a CD73 independent way (Botta Gordon-Smith et al., 2014).

Adenosine is further metabolized into inosine by ADA. Experiments have shown that the blockade of CD26, a surface molecule that binds ADA in humans, enhances immune suppression by Tregs (Mandapathil et al., 2010). Our findings on the frequency of CD26+ expressing lymphocytes did not support previously published results. Ellingsen had reported that the frequency of CD26+ lymphocytes is similar in peripheral blood and the synovial fluid of JIA patients (Ellingsen et al., 2008). In rheumatoid arthritis, CD26+ CD3+ lymphocytes even accumulate in the synovial fluid (Mizokami et al., 1996). Nevertheless, we described a significant decrease in CD26+ lymphocytes in the synovial fluid of JIA patients. Also the subsets of CD8+ $CD26^{high}$ T cells, that we identified as MAIT cells, were significantly decreased in the synovial fluid. Thus, if the concentration of adenosine could be increased therapeutically in the joints of JIA patients, the low frequency of CD26 expressing T cells and consequently the low amount of ADA would be beneficial to prevent further metabolism of adenosine.

The role TCRγδ+ T cells in synovitis

Our data showed that the frequency of TCRγδ+ T cell in the synovial fluid increased in about half of the patients while in the other half we observed a slight decrease compared to peripheral blood. So far there has been now studies on the differentiation (naïve/effector/memory) phenotypes of TCRγδ+ T cell in peripheral blood and the synovial fluid of JIA patients. In contrast to CD4+ and CD8+ lymphocytes, we found a significant increase of naïve and central memory TCRγδ+ T cells and a decrease in the frequency of effector memory and TEMRA TCRγδ+ T cells in the synovia. Interestingly, the frequency of naïve TCRγδ+ T cells varied a lot in the synovial fluid, from 5% to 65%. This could be related to the duration of ongoing inflammation in the joints. Naïve TCRγδ+ T cells might migrate into the joints at an early point of inflammation. This would also explain the decrease of naïve TCRγδ+ T cells in peripheral blood. If children are punctured when inflammation in the joint has

recently started, the frequency of naïve TCRγδ+ T cells might still be high. In contrast to that, we might see a shift to effector memory TCRγδ+ T cells when inflammation has persisted for some time. To support this speculation, further markers of inflammation such as CRP, ESR and anamnestic information about the duration of symptoms would have to be taken in consideration.

Due to the combination of antibodies in our panel we could not discriminate between Vδ1 and Vδ2 TCRγδ+ T cells. However, Vδ2 T cells are mostly CD45RA- whereas the majority of Vδ2- TCRγδ+ T cells are CD45RA+ (Bindszus, unpublished obervations). Hence, the accumulation of what we identified as naive CD45RA+ CD27+ TCRγδ+ T cells in the synovial fluid might also reflect a higher percentage of Vδ2- TCRγδ+ T cells in this compartment.

Similar to TCRαβ+ T cells, we saw a significantly increased frequency of TCRγδ+ cells expressing the activation marker CD69 and of TCRγδ+ T cells lacking CD62L, both features of recent activation. Concerning effector function we found that the frequency of TNFα- and IFNγ-producing TCRγδ+ T cells was similar in peripheral blood and synovial fluid. These findings are consistent with published results (Bendersky et al., 2012). Interestingly, the frequency of IL-17-producing TCRγδ+ T cells was increased in three patients while IL-17 production was similar in both compartments in the other two patients. In contrast to our findings, Bendersky described a decreased frequency of IL-17-producing Vδ1 and Vδ2 T cells in the synovial fluid of all 12 JIA patients analysed (Bendersky et al., 2012). There are several indications that IL-17-producing TCRγδ+ T cells play an important role in autoimmunity. In the mouse model of arthritis, TCRγδ+ T cells are a major producer of IL-17, and depletion of TCRγδ+ T cells leads to less severe arthritis (Roark et al., 2007) (Roark, 2007). However, there is evidence that TCRγδ+ T cells differ in mice and humans. Mice have no equivalent to human semiinvariant Vδ2 cells. The functional properties of human IL-17-producing TCRγδ+ T cells in inflammation still remain unclear. It is speculated that IL-17-producing TCRγδ+ T cells attract neutrophils to the site of inflammation and, therefore, enhance inflammatory responses (Kasten et al., 2010). In our limited cohort of three patients with an increased frequency of IL-17-producing TCRγδ+ T cells we did not observe a significantly increased frequency of neutrophils in these patients compared to others.

Patients treated with biologics show signs of longer disease progression.

At last we wanted to analyze how the treatment with biologics affects leukocyte populations. From the six patients treated with biologics, three were treated with etanercept, one patient was treated with anakinra, which is an IL-1 receptor antagonist, and two patients received canakinumab, which is an antibody against IL-1 beta. In all patients treated with biologics we found a significant increase in matured lymphocytes in peripheral blood. This is unlikely the result of treatment but rather to the fact that biologics are only used in patients with a severe and long progression of disease and, therefore, these patients have had chronic inflammatory processes for longer periods. The frequency of CD69+ CD4+ T cells and IFNγ producing T cells was increased. Nevertheless, the frequencies of TNFα and IL-17-producing CD4+ and CD8+ T cells were decreased. In the three patients treated with etanercept it fits that we found a decreased production of TNFα. Interestingly, patients treated with biologics had an increase of CD39+ Tregs, which may reflect an increased effort of the immune system to control inflammation. Since our cohort only included six patients that were treated with biologics we can only speculate about the effects of these medication group and conclusions have to be evaluated very carefully. A longitudinal follow up on these patients would be required for further clarification of the influence of these powerful drugs on immune function.

How could immunophenotying influence therapeutic decisions and the clinical outcome in JIA patients?

There have been many studies analyzing single effector cells in JIA. However, there has been no study integrating a variety of immune cells and focusing of the phenotype of these cells. In this study we propose an easy and efficient protocol to characterise the most relevant inflammatory and regulatory subsets, including the activation effector and maturation status, in the peripheral blood and the synovial fluid of JIA patients. Since very little material is needed, the characterization could be easily integrated into the clinical practice, during routine laboratory examinations and whenever inflamed joints are punctured. Some effector cells such as regulatory subsets and cytokine producing lymphocytes also contribute to the pathogenesis of other autoimmune diseases, e.g. MS and lupus erythematosus, this protocol could

also be used in these patients' cohorts.

Comparing the leukocyte subsets in the peripheral blood of untreated JIA patients we could not identify lymphocyte profiles that clearly distinguish between healthy donors and JIA patients or different subgroups of JIA. Previous studies also did not find differences in the lymphocyte populations in JIA patients and healthy donors. Thus, lymphocyte profiling of peripheral blood leukocytes does not seem to serve as an indicator to identify JIA patients or those with a more severe progression of disease. However, complete transcriptome analysis might yield more information.

However, our phenotyping of the synovial fluid revealed some interesting results: the frequency of cytokine producing CD4+, CD8+ and TCRγδ+ T cells varied in different patients, indicating a unique cytokine milieu in the inflamed joints of individuals. Thus, therapies that target specific molecules such as TNFα are probably more efficient in those patients with increased amounts of this cytokine. Interestingly the frequency of IL-17-producing cells also varied a lot. We confirmed the increased frequency of IL-17-producing cells by ELISA. IL-17 antibodies as a new therapy option have been used in phase 2 trials in the treatment of psoriasis (Gisondi et al., 2014) and ankylosing spondylitis (Baeten et al., 2013). Based on our finding, they may also be an efficient therapeutic approach in JIA patients with increased IL-17 production. Using our protocol to phenotype immune cells in the synovial fluid prior to the introduction of medication, may serve as a tool to identify the most prominent cytokine driving synovitis in individuals. Thus therapeutic decisions may be optimized. Immunophenotyping could also help to evaluate the duration of ongoing inflammation: increased frequency of naïve TCRγδ+ T cells may be found in patients with only recent synovitis. Thus, the composition of memory subsets of TCRγδ+ T cells may help physicians to evaluate how long synovitis as been active in a patient.

Finally we have also found differences in the frequency of CD39+ lymphocytes that may reflect the amount of ATP in the joint. Further experiments would have to confirm this assumption. Increased frequencies of CD39+ cells may identify patients that have more severe synovitis and hence are in need for a more aggressive therapy. We, as others (Moncrieffe et al., 2010), abide for an insufficient breakdown of ATP in the synovial fluid and propose a new therapy option with the goal to increase to local amount of adenosine. Summarizing, we propose that immunophenotypimg of the synovial fluid in JIA patients could facilitate an individualized therapy. It helps to understand the pathogenesis of JIA in a better way

and may assist to develop new therapy options. A fast and efficient therapy is especially important in JIA patients, since insufficient control of inflammation in the joints leads to a rapid destruction of cartilage and bone, which is extremely undesirable in children that have not completed growth.

SUMMARY

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease in pediatric patients. It has been associated to an exacerbated inflammatory response together with a defect in immune regulation. Most studies in this direction have characterised Th1 and Th17 effector cells, and naturally occurring CD4+ CD25^{high} Tregs. Using multiparameter flow cytometry we have extended these studies to include parameters that distinguish recent from chronic activation, other regulatory cell types, innate like lymphocytes and molecules related to ATP degradation. The aim of this study was to establish the existence of different leukocyte profiles to identify patients with a more severe disease progression.

Analysis of peripheral blood (PB) from 34 untreated JIA patients showed minimal differences in the lymphocyte composition: The frequency of effector memory TCRγδ+ T cells was significantly increased in the PB of JIA patients. Comparing different entities of JIA we found a decreased frequency of IL-17-producing lymphocytes in patients with oligo- and polyarthritis, while patients with enthesitis-related JIA had a reduced frequency of TNFα-producing T cells. In contrast to PB, the synovial fluid (SF) of nine patients contained significantly higher percentages of activated cells, both recently activated (CD69+ and CD25+) CD4+ and CD8+ T cells, as well as T lymphocytes that have undergone repetitive stimulation (CD4+ CD28-). These cells are clearly pathogenic, as indicated by higher production of IFNγ and TNFα compared to PB. The frequency of IL-17-producing cells, in contrast, was highly elevated in three patients, while one patient showed levels comparable to PB. Interestingly, all regulatory cell subsets analysed (CD4+ CD25^{high} CD127- Tregs, CD39+ Tregs, CD56^{bright} NK cells and CD8+CD28- cells) were found highly represented in the SF, probably reflecting an attempt to control a very persistent chronic inflammatory response. While the number of CD39+ conventional T cells was also elevated in inflammed joints, we found a decrease in CD73+ T cells, indicating that AMP is not metabolized into the immune regulatory molecule adenosine in inflamed joints.

Summarising we developed a quick and easy protocol using flowcytometry to phenotype important leukocyte subsets in the PB and SF of JIA patients. Based on our findings, we cannot use PB to predict disease progression. However in the SF, we found interindividual differences in the frequency of cytokine producing T cells and an impaired ATP metabolism. Hence, immunophenotypic analysis can serve to identify new target sites for modern therapy regimes.

ZUSAMMENFASSUNG

Die juvenile idopathische Arthritis (JIA) ist die häufigste rheumatische Erkrankung im Kindesalter und durch eine gesteigerte Immunantwort sowie einen Defekt in immunregulatorischen Mechanismen charakterisiert. Bisherige Studien konzentrierten sich vor allem auf die Phänotypisierung von Th1, Th17 und Tregs. In unserer Studie haben wir die Analyse um Parameter erweitert, die zwischen kürzlich und chronisch aktivierten Zellen unterscheiden. Des Weiteren haben wir regulatorische Zellpopulationen, sogenannte „innate like“ Lymphozyten und Moleküle, die mit dem Abbau von ATP assoziiert sind, analysiert. Ziel dieser Studie war es herauszufinden, ob man durch die Immunphänotypisierung von peripherem Blut (PB) oder Synovialflüssigkeit (SF) Patienten identifizieren kann, die einen schweren Erkankungsverlauf haben.

Im PB von 38 unbehandelten Patienten mit JIA konnten wir nur minimale Unterschiede zu gesunden Kontrollen finden. Auffallend war die Erhöhung von TCRγδ+ Effektor-Gedächtnis T Zellen im Blut von JIA Patienten. Auch im PB von verschiedenen JIA Untergruppen konnten wir nur geringe Unterschiede in der Häufigkeit von Zytokin produzierenden Lymphozyten feststellen. Patienten mit Oligo- und Polyarthritis zeigten weniger IL-17-produzierende T Zellen als gesunde Kontrollen, während Kinder mit der Enthesitis-assoziierten Form von JIA reduzierte TNFα- produzierende T Zellen aufwiesen.

Im Gegensatz zu PB zeigte sich in der SF von neun Patienten eine Erhöhung von aktivierten CD69+ und CD25+ T Zellen, als auch von wiederholt stimulierten CD62L- T Zellen. Diese Zellen haben einen eindeutig pathogenen Phänotyp, da sie vermeht IFNγ und TNFα produzieren. In drei Patienten zeigte sich eine deutliche Erhöhung von IL-17 in der SF. In einem weiteren Patienten war die Häufigkeit von IL-17-produzierenden Zellen im PB und in der SF vergleichbar. Interessanterweise akkumulierten alle analysierten regulatorischen Zellen (CD4+ $CD25^{hoch}$ CD127- Tregs, CD39+ Tregs, $CD56^{bright}$ NK Zellen and CD8+CD28- Zellen) in der SF. Dies könnte einen Versuch des Immunsystems darstellen, die persistierende chronische Entzündungsreaktion zu kontrollieren. Auch die Anzahl von konventionellen CD39+ T Zellen in der SF war erhöht. Dahingegen war die Häufigkeit von CD73+ T Zellen deutlich reduziert, wodurch die Hydrolyse von AMP zu immunregulatorischen Adenosine beeinträchtigt wird.

Zusammenfassend haben wir in dieser Studie ein einfaches und effizientes Protokoll zur durchflusszytometrischen Phänotypisierung von Leukozyten im PB und in

der SF entwickelt. Unsere Daten zeigen, dass das PB kein geeignetes Material zur Einschätzung des Krankheitsverlaufs von JIA Patienten ist. Jedoch zeigten sich interindividuelle Unterschiede im Zytokinmilieu der SF. Daneben fanden wir Hinweise für einen beeinträchtigten ATP Metabolismus in entzündeten Gelenken. Folglich kann die Immunphänotypisierung von Patientenmaterial neue Therapieziele identifizieren.

ABBREVIATIONS

A2AR	Adenosine 2A receptor
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANA	Anti- nuclear antibodies
APC	Antigen presenting cell
ATP	Adenosine triphosphate
CCP	Cyclic citrullinated peptide
CCR	Chemokine receptor
CD	Cluster of differentiation
CM	Central memory
DMARDs	Disease modifying anti- rheumatic drugs
EA	Enthesitis-associated arthritis
EM	Effector memory
g	Earth's gravitational acceleration
HD	Healthy donors
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
IL	Interleukin
JIA	Juvenile idiopathic arthritis
MAIT cells	Mucosal asscociated invariant T cells
MIF	Macrophage migration inhibitory factor
MS	Multiple sclerosis
NK cells	Natural Killer cells
NKT	Natural Killer T cells
NSAIDs	Non-steroidal anti-inflammatory drug
OA	Oligoarthritis
OS	Psoriatic arthritis
PA	Polyarthritis
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RF	Rheumatoid factor
RT	Room temperature
sJIA	Systemic JIA
SF	Synovial fluid
TCR	T cell receptor
TEMRA	Terminally differentiated effector memory T cells
TGFβ	Transforming growth Factor beta
Th	T helper cell
TNFα	Tumor necrosis factor alpha
Tregs	Regulatory T cells
VTCN1	V-set domain containing T cell activation inhibitor 1

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APPENDIX

Appendix 1 Information for parents

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INFORMATIONSBLATT

Stand 06.05.2011

Aufklärungsbogen für die wissenschaftliche Studie
"Studie der immunpathologischen Mechanismen in juveniler idiopathischer Arthritis “

Gewinnung und Aufbewahrung von Blut und Gelenkflüssigkeit im Rahmen ergänzender wissenschaftlicher Untersuchungen

(beraten von der Ethik-Kommission der Ärztekammer Hamburg am 01.03.2011,
Autor: Dr. Eva Tolosa

Lieber Patient, liebe Eltern,

etwa 20.000 Kinder leiden in Deutschland an juveniler idiopathischer Arthritis (JIA).
Warum junge Menschen an JIA erkranken ist noch nicht vollständig geklärt. Man nimmt an, dass eine genetische Veranlagung zusammen mit einem äußeren Einfluss zu einer sogenannten Autoimmunreaktion führt. Das Abwehrsystem greift dabei körpereigenes Gewebe an und löst eine Entzündung aus.

Im Falle der JIA betrifft diese Entzündung das Gelenk. Mehrer Zellen spielen hierbei eine Rolle, insbesondere weiße Blutkörperchen (Lymphozyten und Makrophagen). In der Gruppe der Lymphozyten gibt es eine besondere Untergruppe, die entzündliche Botenstoffe produzieren, um die Entzündung aufrecht zu erhalten (genannt CD4+ T-Zellen), und andere Zellen die die eine ungewollte überschießende Entzündungsreaktion kontrollieren und unterdrücken (genannt regulatorische T-Zellen). Die genauen Mechanismen, die zur Entstehung der so eben erklärten überschießenden Entzündungsreaktion führen, sind größtenteils ungeklärt. Je besser diese Mechanismen erforscht und verstanden werden, desto wirksamere Medikamente können gegen die JIA entwickelt werden.

Unser Ziel ist es zusammen mit Dir/mit Ihnen das Verständnis des Zusammenspiels der Entzündungszellen zu verbessern und mehr über die Entstehung von Autoimmunerkrankungen zu lernen. Von unseren Ergebnissen erhoffen wir uns zur Entwicklung besserer Medikamente gegen die JIA beizutragen.

Was haben wir vor? - allgemeine Informationen

Dein Arzt hat bei dir/ Ihrem Kind die Erkrankung JIA festgestellt. Für unsere Forschungsprojekt würden wir gerne eine kleine Probe von deinem Blut/ vom Blut Ihres Kindes und gegebenenfalls eine kleine Menge von deiner Gelenkflüssigkeit/ der Gelenkflüssigkeit Ihres Kindes untersuchen. Das Material wird dann bei uns im Labor intensiv untersucht. Unser Hauptziel ist es die Zellen, die an der oben beschriebenen Entzündung teilhaben, genau zu charakterisieren. Dafür sammeln wir Blut- und Gelenkflüssigkeitsproben von verschiedenen an JIA erkrankten Kindern über einen Zeitraum von 2 Jahren. Diese Ergebnisse vergleichen wir dann mit Zellen aus dem Blut von gesunden Kindern. Dies Untersuchungen und ihre Ergebnisse haben, zum jetzigen Zeitpunkt, keine unmittelbare Bedeutung für deine Behandlung/die Behandlung Ihres Kindes.

Untersuchungsmaterial: ***Blut***

Für unsere Untersuchungen brauchen wir nur wenig Blut, ungefähr so viel wie auf einen halben Teelöffel passt. Das Blutröhrchen wird im Rahmen von Routinekontrolluntersuchungen

abgenommen, sodass du/ Ihr Kind nicht extra gestochen wird. Eine Blutentnahme aus rein wissenschaftlichen Gründen erfolgt nicht.

Untersuchungsmaterial: ***Gelenkflüssigkeit***

Aufgrund der JIA sind bei Dir/bei Ihrem Kind eins oder mehrere Gelenke entzündet. Durch die Entzündung hat sich mehr **Gelenkflüssigkeit** gebildet, die manchmal entfernt werden muss. Nach der Entfernung der Gelenkflüssigkeit wird diese, sofern sie nicht für weitere Untersuchungen gebraucht wird, weggeworfen. Du wirst nicht/ Ihr Kind wird nicht aus rein wissenschaftlichen Gründen gelenkpunktiert.

Wir würden uns sehr freuen, wenn wir mit deinem/ Ihrem Einverständnis die zu verwerfende Gelenkflüssigkeit für die Erforschung von Ursachen und Mechanismen der JIA benutzen dürfen.

Was wird gemacht? - geplante Untersuchungen

Untersuchungen an Zellkulturen

Im Rahmen dieses Projektes sind **immunologische Untersuchungen** an T-Zellen und Makrophagen (weiße Blutkörperchen) geplant. Es werden Experimente mit Blutproben und gegebenenfalls Gelenkflüssigkeit durchgeführt, um bestimmte Zellpopulationen (T-Helferzellen, Makrophagen, regulatorische Zellen) und deren Funktion zu untersuchen.

Untersuchungen der Genaktivität und genetische Untersuchungen

In unsere Forschungsarbeit möchten wir unter anderem untersuchen, wie stark das Abwehrsystem ist. Als Maß dafür wie stark und effektiv die Abwehrzellen arbeiten, messen wir Proteine, die von den Zellen hergestellt werden. Aufgrund der gemessenen Menge an Protein können wir untersuchen, ob die Funktion der Zellen normal, eingeschränkt oder verstärkt ist. Dazu vergleichen wir deine Blutprobe/ die Blutproben Ihres Kindes mit Blutproben von Kindern, die nicht unter JIA leiden.

Aufbewahrung der Proben:

Proben (Blut und Gelenkflüssigkeit), die nicht sofort untersucht und verarbeitet werden, werden eingefroren und im Gefrierschrank des Labors für 10 Jahre gelagert. Dies ist wichtig, da

mit zu einem späteren Zeitpunkt Versuche wiederholt und damit bestätigt werden können. Nach 10 Jahren werden die Proben vernichtet.

Zusätzliche Hinweise:

Datenschutz

Die im Rahmen der Studie nach Einverständniserklärung erhobenen persönlichen Daten unterliegen der Schweigepflicht und den datenschutzgesetzlichen Bestimmungen. Sie werden in Papierform und auf Datenträgern in der Poliklinik für Kinder- und Jugendmedizin des Universitätsklinikums Eppendorf Abteilung Kinderrheumatologie aufbewahrt und pseudonymisiert[1] (Erklärung siehe am Ende dieses Bogens) gespeichert.

Die Aufzeichnung bzw. Speicherung erfolgt für die Dauer von 10 Jahren. Bei der Pseudonymisierung (Verschlüsselung) werden der Name und andere Identifikationsmerkmale (z.B. Teile des Geburtsdatums) durch eine mehrstellige Buchstaben- und Zahlenkombination, auch Code genannt, ersetzt, um die Identifizierung der Studienteilnehmer auszuschließen oder wesentlich zu erschweren. Zur Entschlüsselungsliste und zu den Daten haben neben der Studienleiterin (Frau PD Eva Tolosa) nur noch ihre Stellvertreterin (Frau Isabell Baumann) und die an der Studie beteiligten Ärzte (Frau Dr. Weißbarth-Riedel und Frau Dr. Lipovac) Zugang. Die Nutzung der Daten erfolgt nur in pseudonymisierter Form. Eine Weitergabe der erhobenen Daten im Rahmen des Forschungszwecks erfolgt nur in pseudonymisierter Form. Gleiches gilt für die Veröffentlichung der Studienergebnisse.

Sie haben das Recht, über die personenbezogenen Daten Ihres Kindes Auskunft zu verlangen, und über möglicherweise anfallende personenbezogene Ergebnisse der Studie informiert oder nicht informiert zu werden. Gegebenfalls wird der Leiter der Studie Ihre Entscheidung darüber einholen.

Die Studie ist durch die zuständige Ethik-Kommission beraten worden. Der zuständigen Landesbehörde kann ggf. Einsichtsnahme in die Studienunterlagen gewährt werden. Sobald der Forschungszweck es zulässt wird der Schlüssel gelöscht und die erhobenen Daten damit anonymisiert[2]

Entscheidungsfreiheit

Eine Einwilligung kann jederzeit ohne Angabe von Gründen widerrufen werden, ohne dass Ihrem Kind daraus Nachteile bezüglich einer laufenden oder zukünftigen Behandlung entstehen. Bei Widerruf des Einverständnisses werden die bereits erhobenen Daten gelöscht. Ein Widerruf bereits anonymisierter Daten ist nicht möglich.

Besonderere Hinweise:

Die Teilnahme an dieser Studie ist freiwillig.

Es ist kein persönlicher Nutzen mit der Teilnahme verbunden.

Für die im Rahmen dieses Projektes benutzte Blutprobe gilt:

- Das entnommene Gewebe wird *nicht kommerziell* verwendet.
- Es wird *nicht zu therapeutischen Zwecken* bei anderen Menschen verwendet.

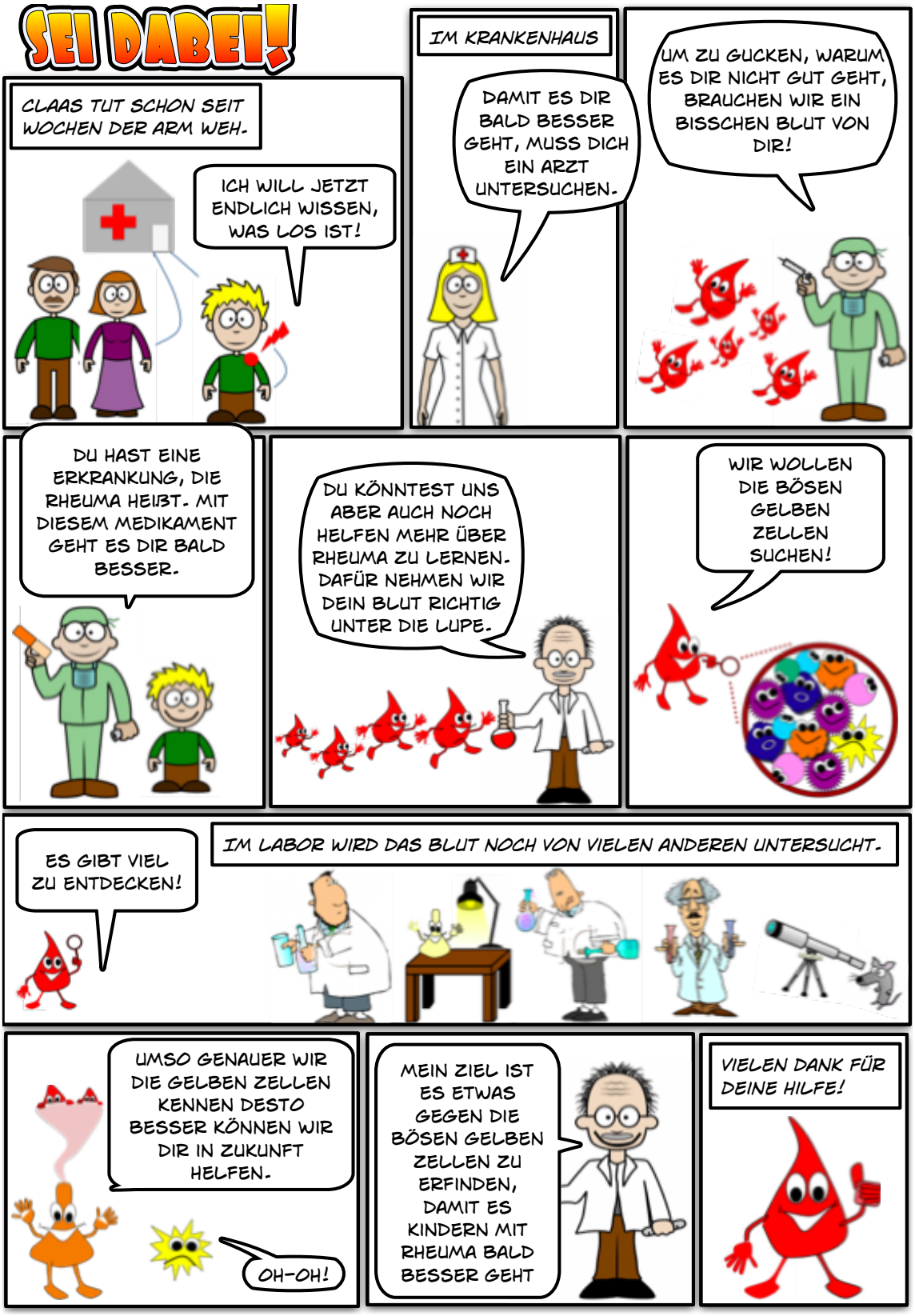
▪ Sollten sich neue wissenschaftliche Fragestellungen ergeben, die mit dem hier beschriebenen Forschungsvorhaben nicht mehr übereinstimmen, für die die gelagerten Gewebeproben aber von großem Nutzen wären, werden Sie zum gegebenen Zeitpunkt gesondert um eine erneute Einwilligung gebeten.

Vielen Dank für die Unterstützung unseres Forschungsprojektes!

[1]Pseudonymisieren ist das Ersetzten des Namens und anderer Identifikationsmerkmale durch ein Kennzeichen zu dem Zweck, die Bestimmung des Betroffenen auszuschließen oder wesentlich zu erschweren (§ 3 Abs. 6a BDSG). Der in diesem Zusammenhang benutzte numerische Code setzt sich wie folgt zusammen: JIA001_xF/M, JIA002_xF/M usw. Dabei steht JIA für juvenile idiopathische Arthritis, anstelle des x wird das Alter des Kindes eingetragen und durch F (=female/weiblich) bzw. M (=male/männliche) ergänzt.

[2] Anonymisieren ist das Verändern personenbezogener Daten derart, dass die Einzelangaben über persönliche oder sachlich Verhältnisse nicht mehr oder nur mit einem unverhältnismäßig großen Aufwand an Zeit, Kosten und Arbeitskraft einer bestimmten oder bestimmbaren natürlichen Person zugeordnet werden können (§ 3 Abs. 6 Bundesdatenschutzgesetz)

Appendix 2 Comic for children



Appendix 3 Information for children

Informationsblatt für Kinder 6-10 Jahre

Hallo!

20.000 Kinder in Deutschland haben Rheuma. Das sind so viele Kinder, wie in 1000 Schulklassen passen würden. Beim Rheuma spielt dein Abwehrsystem ein bisschen verrückt, weil es statt böse Keime zu bekämpfen dein Gelenk angreift. Warum das so ist, wissen wir noch nicht.

Wie du im Comic siehst, möchten wir gerne ein paar Tropfen Blut von dir extra im Labor untersuchen. Im Labor gucken wir uns die Zellen in deinem Blut und vielleicht auch in deiner Gelenkflüssigkeit genau an. Wichtig dabei ist, dass du für diese zusätzliche Untersuchung nicht extra gestochen wirst.

Uns interessieren bei unserem Projekt vor allem die „bösen Zellen" die dazu führen, dass deine Gelenke manchmal dick und rot werden. So viel ist über diese Schurken nämlich noch nicht bekannt. Deshalb würden wir gerne mit deiner Hilfe mehr über die Merkmale und Eigenschaften dieser Zellen wissen.

Denn wenn wir diese Schurken besser kennen lernen, können wir sie auch besser bekämpfen, damit es dir und anderen Kindern mit Rheuma bald besser geht.

Vielen Dank für deine Hilfe!

Dein Projektteam

Informationsblatt für Kinder 11-14 Jahre

Hallo!

In Deutschland haben ca. 20000 Kinder Rheuma. Wie du sicher weißt, ist das Abwehrsystem bei dir und anderen Kinder, die Rheuma haben, ein bisschen komisch drauf. Anstatt nur böse Keime und Bakterien zu bekämpfen und dich vor Krankheiten zu schützen, greift es manchmal auch deinen eigenen Körper an und löst eine Entzündung aus. Beim Rheuma findet diese Entzündung im Gelenk statt. Immer dann werden diese rot und dick.

Warum der Körper das macht, wissen wir noch nicht. Wir haben aber schon herausgefunden, dass bestimmte Zellen im Blut (sogenannte T-Zellen) dabei eine Rolle spielen. T-Zellen gehören zum Abwehrsystem und sind eigentlich gute Zellen, die Keime bekämpfen. Einige von ihnen machen aber genau das Gegenteil. Sie erkennen Bestandteile von deinem Gelenk und behandeln diese so, als wären sie ein Keim.

In unserem Forschungsprojekt möchten wir mehr über diese Zellen erfahren. Dafür untersuchen wir ein bisschen Blut und vielleicht auch ein bisschen von deiner Gelenkflüssigkeit und suchen diese Zellen zunächst. Anschließend charakterisieren wir die Zellen ganz genau und untersuchen welche Merkmale sie ausmachen. Wenn wir wissen, mit „wem" wir es zu tun haben, können wir bessere Rheumamedikamente entwickeln, damit Kinder mir Rheuma noch besser behandelt werden können.

Vielen Dank für deine Hilfe!

Dein Projektteam

Informationsblatt für Kinder 15-17 Jahre

Hallo!

In Deutschland leiden ca. 20.000 Kinder an Rheuma. Wie du sicher weißt, ist Rheuma eine Autoimmunerkrankung. Das heißt, dass das Immunsystem anstatt nur Keim zu bekämpfen auch körpereigene Bestandteile angreift und eine Entzündung auslöst. Warum einige Kinder an Autoimmunerkrankungen leiden, wissen wir noch nicht.

Bei der ausgelösten Entzündung spielen mehrere Zellen des Abwehrsystems eine Rolle, vor allem weiße Blutkörperchen. Diese bestehen aus zwei Gruppen: Einige Zellen lösen die Entzündung aus, und andere Zellen sind dafür da die Entzündung zu kontrollieren und abzumindern. In unserem Forschungsprojekt setzen wir uns intensiv mit den Zellen des Immunsystems auseinander. Dafür benötigen wir eine kleine Menge Blut und eventuell auch ein bisschen Gelenkflüssigkeit.

Die Proben kommen anonymisiert zu uns ins Labor. Das heißt, dass die Menschen, die mit dem Blut und der Gelenkflüssigkeit arbeiten, nicht wissen wer du bist. Das ist vor allem wichtig, um deine Privatsphäre zu schützen. Nur die Ärzte und die Projektleiterin können die Proben zu deinem Namen zuordnen. Material (also Blut und Gelenkflüssigkeit), welches wir nicht gleich untersuchen können, wird eingefroren und 10 Jahre aufbewahrt. Wir machen da, damit wir auch die Möglichkeit haben einige Versuche zu einem späteren Zeitpunkt zu wiederholen.

Uns interessieren bei unserem Forschungsprojekt vor allem die Anzahl der verschiedenen weißen Blutkörperchen im Blut von Kindern mit Rheuma und die Merkmale dieser Zellen. Dafür charakterisieren wir die Immunzellen und untersuchen ihre Funktion. Unser Ziel ist es mit deiner Hilfe mehr über die Zellen herauszufinden, die beim Rheuma eine Rolle spielen. Wir hoffen, dass unsere Ergebnisse zur Entwicklung von besseren Medikamenten gegen Rheuma beitragen.

Vielen Dank für deine Hilfe!

Dein Projektteam

Appendix 4 Consent form

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22763 Hamburg
Telefon: 040 / 88908 - 398
Telefax: 040 / 88908 - 386
sandra.lipovac@kinderkrankenhaus.net

EINWILLIGUNGSERKLÄRUNG

Name des Kindes:

Name des Erziehungsberechtigens:

Stand 03.04.2011

FORSCHUNGSPROJEKT

"Studie der immunpathologischen Mechanismen in juveniler idiopathischer Arthritis “

Einwilligungserklärung zur Gewinnung und Aufbewahrung von Blutproben und/oder Gelenkflüssigkeit für wissenschaftliche Untersuchungen.

(beraten von der Ethik-Kommission der Ärztekammer Hamburg am 01.03.2011)
Autor: Eva Tolosa

Erklärung des Patienten/des Erziehungsberechtigten nach dem Aufklärungsgespräch mit dem

Arzt Frau / Herr Dr. …….....

hat mir den Zweck und die Vorgehensweise für die Verwendung von einer Blutprobe und/oder Gelenkflüssigkeit für wissenschaftliche Untersuchungen im Rahmen des For

schungsprojektes „Immunregulatorische Mechanismen in Autoimmunerkrankungen“ ausführlich und verständlich dargelegt.

Das Ziel der Studie ist zusammen mit Dir/mit Ihnen das Verständnis des Zusammenspiels der Entzündungszellen in JIA zu verbessern. Für die Studie sammeln wir Blutproben und/oder Gelenkflüssigkeit über einen Zeitraum von 2 Jahren. Das Material wird intensiv im Labor analysiert (Einzelheiten siehe Informationsblatt). Am Ende der Studie hoffen wir eine detailliertere Vorstellung von den an der rheumatischen Entzündung beteiligten Zellen zu haben und so zur Entwicklung besserer Medikamente gegen die JIA beizutragen.

Ich habe die Patienteninformation gelesen. Alle mich interessierenden Fragen wurden hinreichend beantwortet. Die zusätzlichen Hinweise ***(freiwillige Teilnahme, kein persönlicher Nutzen, keine kommerzielle Verwertung des Blutes und/oder der Gelenkflüssigkeit, Wahrung des Datenschutzes***) habe ich zur Kenntnis genommen. Eine **Kopie des Infoblatts und dieser Einverständniserklärung** wurde mir ausgehändigt.

Einwilligungserklärung:

□ Ich willige hiermit in die **Benutzung einer Blutprobe meines Kindes** zu wissenschaftlichen Zwecken ein.

□ Ich willige hiermit in die **Benutzung von Gelenkflüssigkeit meines Kindes** zu wissenschaftlichen Zwecken ein.

Im Rahmen dieses Projektes sind **immunologische Untersuchungen** an T-Zellen und Makrophagen (weiße Blutkörperchen) geplant. Es werden Experimente mit Blutproben und Gelenkflüssigkeit durchgeführt, um bestimmte Zellpopulationen (T-Helferzellen, Makrophagen, regulatorische Zellen) und deren Funktion zu untersuchen. Außerdem werden Experimente zur Effektivität der Abwehrzellen durchgeführt. Weitere Einzelheiten sind in der Patienteninformation beschrieben.

Zusätzliche Hinweise:

Datenschutz

Die im Rahmen der Studie nach Einverständniserklärung erhobenen persönlichen Daten unterliegen der Schweigepflicht und den datenschutzgesetzlichen Bestimmungen. Sie werden in Papierform und auf Datenträgern in der Poliklinik für Kinder- und Jugendmedizin des Universitätsklinikums Eppendorf Abteilung Kinderrheumatologie aufbewahrt und pseudonymisiert[1] (Erklärung siehe am Ende dieses Bogens) gespeichert.

Die Aufzeichnung bzw. Speicherung erfolgt für die Dauer von 10 Jahren. Bei der Pseudonymisierung (Verschlüsselung) werden der Name und andere Identifikationsmerkmale (z.B. Teile des Geburtsdatums) durch eine mehrstellige Buchstaben- und Zahlenkombination, auch Code genannt, ersetzt, um die Identifizierung der Studienteilnehmer auszuschließen oder wesentlich zu erschweren. Zur Entschlüsselungsliste und zu den Daten haben neben der Studienleiterin (Frau PD Eva Tolosa) nur noch ihre Stellvertreterin (Frau Isabell Baumann) und die an der Studie beteiligten Ärzte (Frau Dr. Weißbarth-Riedel und Frau Dr. Lipovac) Zugang. Die Nutzung der Daten erfolgt nur in pseudonymisierter Form. Eine Weitergabe der erhobenen Daten im Rahmen des Forschungszwecks erfolgt nur in pseudonymisierter Form. Gleiches gilt für die Veröffentlichung der Studienergebnisse.

Sie haben das Recht, über die personenbezogenen Daten Ihres Kindes Auskunft zu verlangen, und über möglicherweise anfallende personenbezogene Ergebnisse der Studie informiert oder nicht informiert zu werden. Gegebenfalls wird der Leiter der Studie Ihre Entscheidung darüber einholen.

Die Studie ist durch die zuständige Ethik-Kommission beraten worden. Der zuständigen Landesbehörde kann ggf. Einsichtsnahme in die Studienunterlagen gewährt werden. Sobald der Forschungszweck es zulässt wird der Schlüssel gelöscht und die erhobenen Daten damit anonymisiert[2]

Entscheidungsfreiheit

Eine Einwilligung kann jederzeit ohne Angabe von Gründen widerrufen werden, ohne dass Ihrem Kind daraus Nachteile bezüglich einer laufenden oder zukünftigen Behandlung entstehen. Bei Widerruf des Einverständnisses werden die bereits erhobenen Daten gelöscht. Ein Widerruf bereits anonymisierter Daten ist nicht möglich.

Besonderere Hinweise:

Die Teilnahme an dieser Studie ist freiwillig.

Es ist kein persönlicher Nutzen mit der Teilnahme verbunden.

Für die im Rahmen dieses Projektes benutzte Blutprobe gilt:

- Das entnommene Gewebe wird *nicht kommerziell* verwendet.
- Es wird *nicht zu therapeutischen Zwecken* bei anderen Menschen verwendet.
- Sollten sich neue wissenschaftliche Fragestellungen ergeben, die mit dem hier beschriebenen Forschungsvorhaben nicht mehr übereinstimmen, für die die gelagerten Gewebeproben aber von großem Nutzen wären, werden Sie zum gegebenen Zeitpunkt gesondert um eine erneute Einwilligung gebeten.

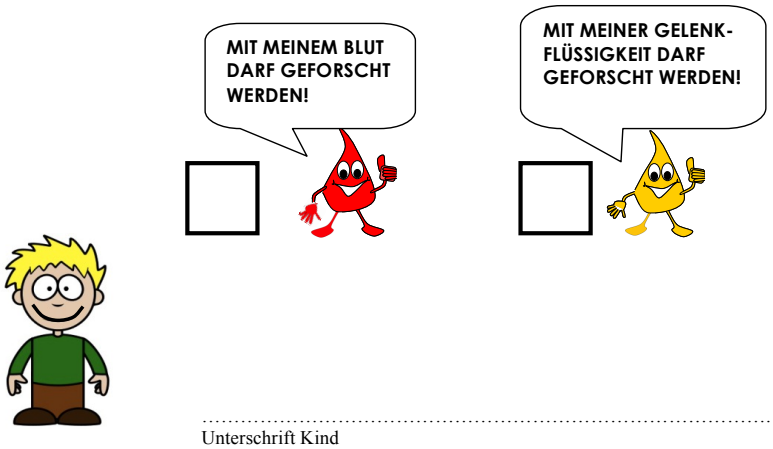
Alle Fragen, die ich gestellt habe, wurden beantwortet.

Durch meine Unterschrift erkläre ich meine Zustimmung zur Benutzung von einer Blutprobe und/oder von Gelenkflüssigkeit meines Kindes für diese Studie.

Vielen Dank für die Unterstützung unseres Forschungsprojektes!

Ihr Studienteam

Hamburg, den ....
Datum Unterschrift Erziehungsberechtigter Unterschrift Arzt



.....
Unterschrift Kind

[1]Pseudonymisieren ist das Ersetzten des Namens und anderer Identifikationsmerkmale durch ein Kennzeichen zu dem Zweck, die Bestimmung des Betroffenen auszuschließen oder wesentlich zu erschweren (§ 3 Abs. 6a BDSG). Der in diesem Zusammenhang benutzte numerische Code setzt sich wie folgt zusammen: JIA001_xF/M, JIA002_xF/M usw. Dabei steht JIA für juvenile idiopathische Arthritis, anstelle des x wird das Alter des Kindes eingetragen und durch F (=female/weiblich) bzw. M (=male/männliche) ergänzt.

[2] Anonymisieren ist das Verändern personenbezogener Daten derart, dass die Einzelangaben über persönliche oder sachlich Verhältnisse nicht mehr oder nur mit einem unverhältnismäßig großen Aufwand an Zeit, Kosten und Arbeitskraft einer bestimmten oder bestimmbaren natürlichen Person zugeordnet werden können (§ 3 Abs. 6 Bundesdatenschutzgesetz)

Appendix 5 Ethics approval

ETHIK-KOMMISSION DER
ÄRZTEKAMMER
HAMBURG
Körperschaft des öffentlichen Rechts

Ärztekammer Hamburg · Postfach 76 01 09 · 22051 Hamburg

Frau
Dr. Eva Tolosa
Institut für Immunologie
Zentrum für Diagnostik
Universitätsklinikum Hamburg-Eppendorf
Martinistr. 52
20246 Hamburg

13.05.2011

Bearb.-Nr.: **PV3746 (Bitte stets angeben!)**
Studie: „Studie der immunpathologischen Mechanismen in juveniler idiopathischer Arthritis“

Sehr geehrte Frau Dr. Tolosa,

über Ihr oben bezeichnetes, zur Primärberatung vorgelegtes Projekt hat die Ethik-Kommission ausführlich beraten.

Das Vorhaben entspricht den berufsrechtlichen bzw. gesetzlichen Anforderungen. Die Ethik-Kommission stimmt dem Vorhaben zu.

Die Kommission weist darauf hin, dass die Verantwortung des Versuchsleiters für das Forschungsvorhaben und seine Durchführung durch das obige Votum der Kommission nicht berührt wird.

Sie werden gebeten, die Ethik-Kommission über alle schwerwiegenden oder unerwarteten Ereignisse, die während der Studie auftreten und die die Sicherheit der Studienteilnehmer gefährden, in Verbindung mit Ihrer Stellungnahme zu unterrichten.
Die Kommission geht davon aus, dass die personenbezogenen Daten der Probanden/ Patienten den datenschutzrechtlichen Vorschriften entsprechend behandelt werden.

Die Ethik-Kommission erwartet, dass ihr nach Abschluss des Projektes unaufgefordert ein Abschluss-Bericht übersandt wird (unter Angabe der Bearb.-Nr.), aus dem der Erfolg/Misserfolg der Studie sowie Angaben darüber, ob die Studie abgebrochen oder geändert bzw. ob Regressansprüche geltend gemacht wurden, ersichtlich sind.

Mit verbindlicher Empfehlung
Im Auftrage der Kommission:

Prof. Dr. med. Th. Weber
- Vorsitzender -

P.S. Die Ethik-Kommission arbeitet auf der Grundlage deutschen Rechts und Berufsrechts sowie in Anlehnung an die ICH-GCP

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BIC: DAAEDEDD, IBAN: DE71 3006 0601 0001 3461 13

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Appendix 6 List of healthy donors and JIA patients

Patient ID	Age	Diagnosis	Medication	Material
CG001	9,3	HD	-	PB
CG002	9,3	HD	-	PB
CG003	10,0	HD	-	PB
CG004	10,0	HD	-	PB
CG005	16,8	HD	-	PB
CG006	11,9	HD	-	PB
CG007	13,9	HD	-	PB
CG008	8,3	HD	-	PB
CG009	3,9	HD	-	PB
CG010	14,6	HD	-	PB
CG011	10,8	HD	-	PB
CG012	7,2	HD	-	PB
CG013	14,6	HD	-	PB
CG014	10,5	HD	-	PB
CG015	12,9	HD	-	PB
JIA002	7,6	OA	-	PB, SF
JIA002_2	7,8	OA	NSAIDs	PB
JIA003	6,1	OA	NSAIDs	PB
JIA003_2	6,2	OA	NSAIDs	PB, SF
JIA003_3	6,3	OA	NSAIDs	PB
JIA004	3,3	OA	MTX	PB
JIA005	17,7	EA	NSAIDs	PB
JIA006	16,6	EA	Eternercept, Sulfasaalzin, NSAIDs	PB
JIA006_2	17,7	EA	Eternercept, Sulfasaalzin, NSAIDs	PB
JIA006_3	18,0	EA	Eternercept	PB
JIA007	13,2	EA	NSAIDs	PB
JIA008	6,4	Sys	Anakinra, MTX, Steroids	PB
JIA008_2	6,8	Sys	Anakinra, MTX	PB
JIA008_3	7,0	Sys	Anakinra, MTX	PB
JIA009	4,1	OA	NSAIDs	PB, SF
JIA009_2	4,5	OA	NSAIDs	PB, SF
JIA009_3	4,0	OA	MTX, NSAIDs	PB
JIA010	12,1	OA	MTX	PB
JIA011	8,3	OA	Eternercept, MTX	PB
JIA012	5,9	EA	NSAIDs	PB
JIA013	14,8	EA	-	PB
JIA014	3,5	Ps	MTX, NSAIDs	PB
JIA015	23,9	EA	Eternercept	PB
JIA016	12,0	PA	-	PB, SF
JIA019	16,0	PA	-	PB
JIA020	11,0	PA	-	PB
JIA021	3,0	PA	-	PB
JIA022	10,1	OA	-	PB
JIA022_2	10,4	OA	NSAIDs, MTX	PB
JIA023	14,9	EA	Sulfasalazin	PB
JIA024	7,0	PA	NSAIDS	PB, BM
JIA025	12,2	OA	MTX, NSAIDs	PB
JIA025_2	12,4	OA	MTX, NSAIDs	PB, SF
JIA025_3	12,0	OA	MTX, NSAIDs	PB

JIA026	17,3	EA	NSAIDs, MTX	PB
JIA026_2	17,0	EA	MTX, NSAIDs	PB
JIA027	14,8	PA	NSAIDs	PB
JIA028	14,0	EA	-	PB
JIA029	16,6	PA	-	PB
JIA030	10,1	OA	-	PB
JIA031	7,4	OA	-	PB, SF
JIA032	17,4	PA	-	PB
JIA033	18,4	PA	-	PB
JIA034	5,9	PA	NSAIDs	PB, SF
JIA035	-0,2	Sys	Canakinumab, MTX	PB
JIA036	14,4	PA	-	PB
JIA037	15,4	PA	-	PB
JIA038	12,8	Sys	Canakinumab, Steroids	PB
JIA039	17,4	PA	-	PB
JIA040	13,4	PA	-	PB
JIA043	10,7	OA	MTX	PB
JIA044	11,4	PA	-	PB
JIA045	8,5	OA	-	PB
JIA046	17,5	OA	-	PB
JIA047	15,5	PA	-	PB
JIA048	12,5	PA	-	PB
JIA049	3,5	OA	MTX	PB, SF
JIA050	13,5	PA	-	PB
JIA051	7,5	PA	MTX	PB
JIA052	11,6	OA	MTX	PB
JIA053	5,5	OA	-	PB
JIA054	12,5	PA	MTX	PB, SF
JIA055	17,2	EA	NSAIDs	PB
JIA056	15,0	OA	NSAIDs	PB
JIA057	6,0	EA	NSAIDs	PB

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Publications

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  Rissiek A, Baumann I, Cuapio A, Dodge- Khatami A, Arck P, Koch-Nolte F, Haag F, Tolosa E
  Journal of Autoimmunity, Volume 58, Pages 12-20
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  Baumann I, Viatour J, Weißbarth-Riedel E., Tzaribachev N, Lipovac S, Sehner S, Tolosa E
  40. Congress of the German Association of Rheumatology, Bochum

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