Aus der Medizinischen Klinik und Poliklinik IV
(Direktor: Prof. Dr. med. Reincke)
Ludwig-Maximilians-Universität München
Sektion für Rheumatologie und Klinische Immunologie
Leiter: Prof. Dr. med. Hendrik Schulze-Koops

Altered T cell plasticity favors Th17 cells in rheumatoid arthritis

Dissertation
zum Erwerb des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

vorgelegt von
Fausto Pirronello
aus Catania – Italien

2018

Gedruckt mit Genehmigung der Medizinischen Fakultät der Ludwig-Maximilians-Universität München

Betreuerin:	Prof. Dr. rer. nat. Alla Skapenko
Zweitgutachter:	Prof. Dr. med. Vigo Heissmeyer
Dekan:	Prof. Dr. Reinhard Hickel
Tag der mündlichen Prüfung:	15.10.2019

Eidesstattliche Versicherung

Pirronello, Fausto

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

Altered T cell plasticity favors Th17 cells in rheumatoid arthritis

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.
Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 19.12.2018 — Ort, Datum

Fausto Pirronello — Unterschrift Doktorand

To Pamela and Matteo

CONTENTS

SUMMARY

Th17 cells are a subset of CD4 effector T cells defined by the production of interleukin 17 (IL-17) and characterized by the expression of the transcription factor RORC. Th17 cells are physiologically involved in the host defense but are also critically implicated in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA), psoriatic arthritis, or multiple sclerosis. In RA, Th17 cells are increased in number and are characterized by an enhanced function. The mechanisms leading to this predominance of Th17 cells in RA are not yet fully understood. Recent studies suggest that Th cell differentiation is a dynamic process enabling effector T cells to switch their phenotype. An altered T cell plasticity might therefore contribute to the shift towards the Th17 phenotype observed in RA (Pirronello et al., Poster Sessions, European Congress of Immunology 2012). In order to test this hypothesis, we analyzed the ability of sorted Th17, Th1 and Th2 cells from RA patients to differentiate into alternative Th subsets. Furthermore, we investigated whether altered levels of expression of key transcription factors and molecules involved in T cell lineage differentiation and stability, as well as the epigenetic status of key cytokine and transcription factor loci, could be involved in the plasticity phenomenon.

In our first experiments, we hypothesized that a supposed difference in plasticity between Th1, Th2 or Th17 derived from healthy donors or RA patients would be due to an intrinsic, possibly genetic, characteristic of the RA Th cell. We therefore isolated CD4 Naïve T cells from the peripheral blood of RA patients and healthy controls (HC) and differentiated them under Th1, Th2, and Th17 inducing conditions. We further purified the cells by performing a cytokine secretion assay and sorting them according to cytokine production into highly pure populations of *in vitro*-differentiated Th1, Th2, and Th17 cells. We then further cultured these cells under different-inducing conditions. RA-derived *in vitro*-differentiated Th cells demonstrated a normal capacity to transdifferentiate under different inducing conditions. Having observed similar plasticity by Th cells generated *in vitro* from HC and RA patients, we hypothesized that an altered plasticity of *in vivo*-generated Th cells might favor the predominance of Th17 cells observed in RA. We therefore isolated CD4 memory cells and further purified them through cytokine secretion assay and sorting to obtain *in vivo*-generated Th1, Th2, and Th17 cells. We then cultured the cells under different -inducing conditions. *In vivo*-generated Th17 cells from RA patients demonstrated a diminished capacity to transdifferentiate into Th1 and Th2 cells, while transdifferentiation of *in vivo*-generated Th1 and Th2 cells towards the Th17 phenotype was enhanced in cells derived from RA compared to those derived from HC.

In order to further investigate these differences, we first analyzed the mRNA expression levels of the master transcription factors of Th17 and Th1 cells RORC and TBX21, in both freshly sorted *in vivo*-generated cells and after transdifferentiation. Freshly sorted Th1 and Th17 cells showed no differences in the expression levels of RORC and T-bet, between RA and HC. Following transdifferentiation, RA patient-derived Th1 cells cultured under different inducing conditions showed a tendency to express higher levels of RORC, compared to cells derived from HC.

Higher levels of RORC expression were also observed in RA patient-derived Th17 cells cultured under Th1 inducing conditions. Meanwhile, the expression levels of TBX21 were lower in RA compared to HC. These results further confirm the enhanced plastic capabilities of RA Th1 cells and the increased resistance towards transdifferentiation of RA patients-derived Th17 cells.

Recent studies suggest an important role for the SGK1-FOXO1-IL23R pathway in Th17 cell differentiation and lineage commitment. We observed an increased expression of SGK1 and IL-23R in both freshly sorted and trandifferentiated Th1 and Th17 cells from RA patients compared to HC. The increased expression of IL23R in RA Th1 and Th17 cells could potentially confer Th1 cells an “advantage” over HC-derived Th1 cells towards Th17 transdifferentiation, while also granting Th17 cells with an increased stability, making them more resistant to shifts towards other Th cell subtypes. An enhanced SGK1-FOXO1-IL23R pathway could therefore contribute to the altered plasticity of Th1 and Th17 cells observed in RA, and to the prevalence of Th17 cells observed in the disease.

Epigenetic modifications of the histones are involved in gene expression control and T cell lineage commitment. To investigate whether such modifications could be involved in the observed altered plasticity of RA Th cells, we analyzed the transcription factor loci RORC and TBX21, in RA- and HC-derived, *in vivo*-generated Th1 and Th17 cells. We found no differences between cells originating from HC and those from RA, indicating that the altered plasticity of RA *in vivo*-generated Th cells is likely not imprinted at the histone modifications level. However, such differences between cells derived from RA and HC might arise upon repeated stimulations in an environment characterized by a cytokine milieu capable of promoting a shift of these cells towards a different Th subtype.

In summary we demonstrated plasticity in both *in vitro*- and *in vivo*-generated human Th cells. We further demonstrated an altered plasticity of RA *in vivo*-derived-Th cells. These phenomena lead to a predominance of cells belonging to the Th17 phenotype, characteristic for the disease. Differences between cells derived from RA and HC in the expression levels of key transcription factors such as RORC and TBX21, as well as components of the SGK1-

FOXO1-IL23R pathway, could contribute to the observed altered plasticity phenomenon. Interestingly, such differences seem not to be epigenetically imprinted at the histone level.

ZUSAMMENFASSUNG

Th17-Zellen sind eine Untergruppe von CD4-Effektor-T-Zellen, die durch die Produktion von Interleukin 17 (IL-17) und durch die Expression des Transkriptionsfaktors RORC charakterisiert sind. Sie sind an der Host-Verteidigung, sowie aber auch an der Pathogenese von Autoimmunkrankheiten wie rheumatoider Arthritis (RA), Psoriasisarthritis oder Multiple Sklerose beteiligt. In RA sind Th17-Zellen erhöht und zeichnen sich durch eine überaktivierte Funktion aus. Die Mechanismen hierfür sind noch nicht vollständig verstanden. Aktuelle Studien deuten darauf hin, dass Th-Zell-Differenzierung ein dynamischer Prozess ist, durch den Effektor T-Zellen ihre verschiedenen Phänotypen erreichen können. Eine veränderte T-Zell-Plastizität könnte daher zur Verschiebung zum Th17-Phänotyp beitragen, welcher in RA beobachtet wird (Pirronello et al., Poster Sessions, European Congress of Immunology 2012). Um diese Hypothese zu verifizieren, analysierten wir die Fähigkeit von Th17-, Th1- und Th2-Zellen von RA-Patienten, sich in alternative Th-Untergruppen zu differenzieren. Darüber hinaus überprüften wir, ob eine veränderte Expression von den wichtigsten Transkriptionsfaktoren und Molekülen, die an der T-Zelllinien-Differenzierung und -Stabilität beteiligt sind, sowie der epigenetische Status von den wichtigsten Zytokinen und Transkriptionsfaktor-Loci in das "Plastizität Phenomen" beteiligt sein könnten. In unseren ersten Versuchen haben wir spekuliert, dass ein Unterschied in der Plastizität zwischen Th1, Th2 oder Th17 von gesunden Spendern oder RA-Patienten, auf eine intrinsische, möglicherweise genetische Eigenschaft der RA Th-Zellen zurückzuführen wäre. Wir isolierten daher CD4 Naive T-Zellen aus dem peripheren Blut von RA-Patienten und gesunden Kontrollen (healthy controls, HC) und differenzierten sie unter Th1-, Th2- und Th17-induzierenden Bedingungen. Die Zellen wurden weiter purifiziert, indem wir einen Zytokin-Sekretionstest durchführten und nach Zytokin-Produktion in hochreine Populationen von *in vitro*-differenzierten Th1-, Th2- und Th17-Zellen sortieren konnten. Wir kultivierten dann die Zellen unter verschiedenen induzierenden Bedingungen. RA-stammenden differenzierten Th-Zellen zeigten eine normale Fähigkeit, sich unter verschiedenen induzierenden Bedingungen, zu transdifferenzieren. Nachdem wir eine ähnliche Plastizität in *in vitro* erzeugten Th-Zellen von HC- und RA-Patienten beobachtet hatten, spekulierten wir, dass eine veränderte Plastizität von i*n vivo* erzeugten Th-Zellen die Dominanz von Th17-Zellen in RA erklären könnte. Wir isolierten daher CD4-Gedächtniszellen und purifizierten sie durch Zytokin-Sekretionstest und Zellsortierung, um *in vivo* erzeugte Th1-, Th2- und Th17-Zellen zu erhalten. Wir kultivierten dann die Zellen unter verschiedenen induzierenden Bedingungen. *In vivo* erzeugte Th17-Zellen von RA-Patienten zeigten eine reduzierte

Fähigkeit, in Th1- und Th2-Zellen transdifferenziert zu werden, während die Transdifferenzierung von *in vivo* erzeugten Th1- und Th2-Zellen in den Th17-Phänotyp in RA-Zellen im Vergleich zu HC-Zellen verstärkt war. Um diese Unterschiede weiter zu untersuchen, haben wir zunächst die mRNA-Expressionsniveaus der wichtigen Transkriptionsfaktoren von Th17- und Th1-Zellen RORC und TBX21 sowohl in frisch sortierten *in vivo*-erzeugten Zellen als auch nach Transdifferenzierung analysiert. Frisch sortierte Th1- und Th17-Zellen zeigten keine Unterschiede in den Expressionsniveaus von RORC und T-bet zwischen RA und HC. Nach der Transdifferenzierung zeigten RA-Th1-Zellen, die unter verschiedenen induzierenden Bedingungen kultiviert wurden, eine Tendenz, RORC höher zu exprimieren, verglichen mit HC-Th1-Zellen. Höhere Expressionsniveaus von RORC wurden auch in RA-Th17 Zellen, die unter Th1-induzierenden Bedingungen kultiviert wurden, beobachtet. Dabei waren die Expressionsniveaus von TBX21 niedriger in RA im Vergleich zu HC. Diese Ergebnisse bestätigen die hohe Plastizität von RA-Th1-Zellen und die erhöhte Resistenz gegen Transdifferenzierung von RA-Th17-Zellen.

Aktuelle Studien zeigen, dass der SGK1-FOXO1-IL23R-Weg, eine wichtige Rolle für die Th17 Zelldifferenzierung und Linienbestimmung spielt. Wir beobachteten eine erhöhte Expression von SGK1 und IL-23R sowohl in frisch sortierten als auch trandifferenzierten Th1- und Th17-Zellen von RA-Patienten im Vergleich zu HC. Die erhöhte Expression von IL23R in RA Th1- und Th17-Zellen könnte Th1-Zellen einen "Vorteil" gegenüber HC Th1-Zellen in der Th17-Transdifferenzierung verleihen, sowie auch Th17-Zellen eine erhöhte Stabilität gewährleisten, wodurch sie resistenter gegenüber Verschiebungen zu anderen Th-Zellen Subtypen sein könnten. Ein überaktivierter SGK1-FOXO1-IL23R-Weg könnte daher zu der veränderten Plastizität von Th1- und Th17-Zellen beitragen, die in RA beobachtet wurden, und auf die Prävalenz von Th17-Zellen, die bei der Erkrankung beobachtet wurde.

Epigenetische Veränderungen der Histone sind an der Genexpressionskontrolle und an der T-Zelllinie-Differenzierung beteiligt. Um zu untersuchen, ob solche Veränderungen an der beobachteten veränderten Plastizität von RA Th-Zellen beteiligt sein könnten, analysierten wir den Transkriptionsfaktor loci RORC und TBX21 in RA- und HC- *in vivo* erzeugten Th1- und Th17-Zellen. Wir fanden keine Unterschiede zwischen HC- und RA-Zellen, was darauf hinweist, dass die veränderte Plastizität von RA *in vivo* erzeugten Th-Zellen wahrscheinlich nicht auf die Histon-Modifikationen geprägt ist. Stattdessen können solche Unterschiede zwischen RA- und HC-Zellen, bei wiederholten Stimulationen in einer Umgebung auftreten, die durch ein Zytokinmilieu gekennzeichnet ist, das in der Lage ist, eine Verschiebung dieser Zellen zu einem anderen Th-Subtyp zu fördern.

Zusammenfassend zeigten wir Plastizität sowohl *in vitro*- als auch *in vivo*-erzeugten humanen Th-Zellen. Des Weiteren konnten wir eine veränderte Plastizität von RA *in vivo*-erzeugten Th-Zellen nachweisen. Diese Phänomene führen zu einer Dominanz der Zellen, die zum Th17-Phänotyp gehören, was charakteristisch für die Krankheit ist. Die Unterschiede zwischen RA- und HC-Zellen in den Expressionsniveaus von wichtigen Transkriptionsfaktoren wie RORC und TBX21 sowie Komponenten des SGK1-FOXO1-IL23R-Weges könnten zu dem beobachteten Plastizitätsphänomen beitragen. Interessanterweise scheinen solche Unterschiede nicht epigenetisch auf Histonebene geprägt zu sein.

1 INTRODUCTION

1.1 T cells in the immune system

The immune system consists of various types of cells and molecules, which have evolved to protect the human body from infectious agents and other harmful substances. The immune system of mammals must fulfill a series of tasks: immunological recognition, immune effector functions, immune regulation and immunological memory. Immunological recognition consists in the ability of the immune system to detect the invading pathogen and activate immune effector functions, which will fight the infection through the concerted action of the blood proteins of the complement system, and antibodies. The pathogen will eventually be destroyed by white blood cells. However, this capacity to fight the infection and determine inflammation must be kept under control through a tight immune regulation. Any failure of such regulation can contribute to the rise of allergies or autoimmune diseases. Finally, higher organisms are characterized by the ability to develop an immunological memory, which allows them to fight recurring pathogens more rapidly and efficiently. This characteristic of the immune system is exploited to artificially induce immunity in animals and humans through vaccination in order to prevent the effects and the spread of several infections (Alberts et al., 2007).

The immune system of higher organisms such as mammals has evolved to include several lines of defense. The first defense mechanisms against infectious agents are physical and chemical barriers such as the skin enclosing the organism and antimicrobial proteins secreted at mucosal surfaces. Furthermore, the immune system consists of two distinct but co-operating branches: the innate and the adaptive immune system. Innate immune responses act rapidly as soon as the first line of defense, represented by external or internal epithelia and mucosal surfaces, is breached (Murphy, 2012). Bacterial cell walls are digested by antimicrobial enzymes, for example lysozymes, while antimicrobial peptides such as defensins lyse the bacterial cell membranes (Ganz T., 2003). At the same time, plasma proteins of the complement system target pathogens for successive lysis or phagocytosis by innate immunity cells such as macrophages (Aderem and Underhill, 1999). In a second phase of the innate immune response, these cellular components of innate immunity are able to recognize surface molecules typical of invading microbes called pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). Innate immune responses act therefore as a first line of defense towards invading pathogens but are incapable of generating an immunological memory. Clearance of pathogens by the immune systems is typically achieved

during the first 96 hours from the initial response. However, if the infection persists it will trigger an adaptive immune response (Murphy, 2012).

The adaptive immune response is activated once the invading pathogen has overcome the defenses laid by the innate immune responses. The most prominent feature of adaptive immunity is its ability to generate immunological memory. Immunological memory consists in the storing, within specific long-lived cells, of the information regarding the specific pathogen that infected the body. This allows for a more rapid clearance of the pathogen upon subsequent infections. Numerous cell types are involved in adaptive immune responses, of both lymphoid origin, such as lymphocytes, and myeloid origin, such as dendritic cells and macrophages. The fulcrum of innate immunity is however mainly represented by lymphocytes. Lymphocytes are able to circulate in the lymphatic system and in the blood stream but are generated and reside mainly within primary, such as bone marrow and thymus, and secondary lymphoid organs such as spleen, lymph nodes and mucosal lymphoid tissues in the gut (Murphy, 2012). There are two main types of lymphocytes: B cells and T cells. B cells are generated in the bone marrow from the common lymphoid progenitor and mature within this organ. These cells, once activated by T helper cells, are able to produce antibodies directed against a specific antigen. They can further differentiate into memory B cells or plasma cells, which will produce large amounts of specific antibodies. T cells, such as CD8 and CD4 T cells, are generated from the common lymphoid progenitor in the bone marrow but mature in the thymus, hence the name. They are able to act as effector T cells for the cytotoxicity of pathogen-infected cells (CD8 T cells) or as "helpers" in activating B cells (CD4 T cells). Most importantly, they are capable of maintaining memory of the antigen they encountered. Maturation of T cells, also called at this stage thymocytes, occurs in the thymus, a primary lymphoid organ found in the upper chest of humans (Luckheeram et al. 2012). Upon arrival to the thymus these precursors are devoid of most of the surface molecules characteristic of mature T cells. They are in particular lacking expression of both CD4 and CD8 and are therefore named double-negative thymocytes. At this stage cells can branch into two different directions which lead to the development of either γ:δ T cells or, more frequently α:β T cells (Lauritsen et al., 2006). Through the so-called V(D)J recombination, thymocytes are able to express α:β T cell receptors, which confer antigen specificity to each cell, and ability to co-express both CD4 and CD8 on their surfaces, in a phase called double positive stage. CD4/CD8 double positive cells migrate deep within the thymic cortex where they will survive only a few days unless they are rescued by the engagement of their TCR by a MHC complex carrying a self-peptide, in a process called positive selection (Hogquist et al., 1997). While only 10-30% of the double positive cells survive, they have to also undergo a

negative selection, in which apoptosis is induced in those cells that interact too strongly with self-peptides presented to these cells, thereby eliminating potentially self-reactive cells which could cause autoimmune reactions (Surh and Sprent, 1994; Starr et al., 2003). MHC class I and MHC class II are necessary for the development of CD8 and CD4 T cells, respectively and are required for positive selection. What triggers the lineage choice is the strength of the Lck signal given by the co-receptor. When the TCR and the co-receptor of a thymocyte encounter a MHC molecule, it triggers first a downregulation of both CD4 and CD8, followed by re-expression of CD4 and low levels of CD8. This configuration of CD4+ CD8 low thymocytes manifests itself independent of whether the contact happens with a MHC class I or II. If the cell is bound by a MHC class II molecule then the re-expression of CD4 leads to a stronger Lck signal which further promotes differentiation along the CD4 pathway, with a complete downregulation of CD8. If instead the cell is in contact with a MHC class I molecule, re-expression of CD4 does not lead to a strong Lck signaling which in turn determines CD4 downregulation and CD8 commitment (Singer et al., 2008). Most thymocytes develop into either CD4 or CD8 T cells, however those cells that receive a stronger signal by a self-antigen than that which leads to CD4 lineage commitment, but not strong enough to induce cell death, tend to upregulate expression of the transcription factor Forkhead-box P3 (FoxP3) and therefore, development into regulatory T cells (Tregs). Tregs express high levels of CD25 and CTLA-4; they are responsible for the suppression of inflammation and are essential to control immune responses and prevent auto-immune reactions (Zheng and Rudensky, 2007). Mature naive CD4 and CD8 T cells are drawn to the bloodstream by the lipid molecule sphingosine 1-phospate (S1P), with which they interact through the G-protein-coupled receptor S1P1. The homing of these cells to the peripheral lymphoid organs is mediated by chemokines (Cyster, 1999, 2005). They can then enter said organs through their expression of the lymph-node homing receptor CD62L (L-selectin) (Rosen, 2004).

1.2 Naïve CD4 T cell differentiation

Naïve T cells in the bloodstream can enter lymphoid organs, such as lymph-nodes, where they are exposed to antigen presenting cells (APCs) carrying MHC-bound peptide molecules corresponding to a plethora of different antigens. Naïve T cells that do not encounter their corresponding antigen, are free to re-enter the bloodstream via the lymphatic system. In case the T cell will bind an APC carrying the corresponding antigen, this cell will cease to migrate, instead undergoing clonal expansion and differentiation, rapidly multiplying and generating a

large number of cells with identical specificity against the invading pathogen. There are three main types of cells capable of presenting antigens to T cells, which can localize to the lymph-node: dendritic cells, macrophages and B-cells. Dendritic cells are specialized in capturing, by phagocytosis, macropinocytosis or direct intake of viruses through the plasmatic membrane, all types of invading pathogens and processing them to present their antigens. Furthermore, dendritic cells are mainly present in T cell areas and are the major drivers of clonal expansion and differentiation of naïve T cells (Guermonprez et al., 2002). On the other hand, macrophages and B-cells are specialized in processing and presenting antigens from intracellular pathogens and soluble antigens, respectively (Unanue, 1984; Pierce et al., 1988; Underhill et al., 1999; Shirota et al., 2002).

When a mature naïve T cell encounters an APC, such as a dendritic cell, it first binds it transiently through the interaction of its surface protein LFA-1 with ICAM-1 and ICAM-2 exposed on the surface of the APC. If the T cells recognizes a peptide:MHC ligand on the surface of a dendritic cell, the surface protein LFA-1 on the T cell undergoes a conformational change that greatly increases its affinity to the surface proteins ICAM-1 and ICAM-2. This binding stabilizes the interaction between T cell and dendritic cell. The T cell then replicates, with its daughter cells also binding the same dendritic cell, expanding and differentiating into effector T cells (Murphy, 2012).

The expansion of T cells largely depends on antigen recognition by the TCR, which determines the expression or activation of the transcription factors NFAT, AP-1 and NF-κB, which in turn promote IL-2 expression. IL-2 is essential for the proliferation and differentiation of T cells and it is produced by the activated T cells themselves. However, a fundamental role is played by co-stimulation through the binding of CD28 on the surface of the CD4 T cell to its ligands CD80 and CD86 on an APC. CD28 signaling, also known as "signal 2", activates PI 3-kinase thereby enhancing the expression of AP-1 and NF-κB, which in turn increases the transcription of IL-2 mRNA (Zhou et al., 2002; Acuto et al., 2003). Furthermore, CD28 signaling is able to prolong the half-life of IL-2 mRNA by promoting the expression of proteins that block an "instability sequence" present on the IL-2 mRNA (Seko et al., 2006). PI 3-kinase also contributes to the activation of Akt, which results in the promotion of growth and survival of the cell, thereby increasing the total IL-2 production by the activated T cell (Eder et al., 1998; Kane et al., 2001).

Apart from their "effector" status, CD4 T cells can also differentiate into functionally different subtypes, which can be classified based on the cytokines they produce, the transcription factors that drive their differentiation and lineage commitment, and the

immunological function that they mediate (Hirahara et al., 2010). These classes of CD4 T cells comprise Th1, Th2, Th9, Th17 and regulatory T cells. Th1 cells are specialized in fighting infections by intracellular bacteria such as mycobacteria and viruses (Agnello et al., 2003). These pathogen are able to resist degradation inside the vesicles of macrophages thereby infecting the cell. Th1 cells are able to recognize bacterial antigens exposed on the surface of the infected macrophages and further enhance the microbicidal activity of the macrophage, which is then able to clear out the invading pathogen. Th1 cells are characterized by their production of IFNγ and the expression of their master transcription factor Tbet. Th2 cells are dedicated to the control of infections by parasites such as helminthes (Mowen et al., 2004). The most important cytokine produced by Th2 cells is IL-4 and the master transcription factor is GATA-3. IL-4 promotes responses mediated by mast cells, eosinophils and determines the class switching in B cells, which leads to IgE production. While IL-4 has in many cases an anti-inflammatory effect, it is involved also in the rise of allergies, which are primarily mediated by IgE antibodies. Additionally, Th2 cells also produce IL-5 and IL-13. Among other factors, also the "strength" of TCR signaling is an important driver of Th1 versus Th2 lineage commitment (Tao et al., 1997; Zhu et al., 2010). Initially Th1 and Th2 cells were considered to be the only two existing subsets of CD4 T cells (Mosmann et al., 1986; 1989; Hirahara et al., 2010). The Th1/Th2 paradigm was however revised upon the discovery of a class of IL-17 producing CD4 T cells, named Th17 cells, which will be described more in detail in the following chapter. Although Interleukin 22 (IL-22) was considered to be produced primarily by Th17 cells, in 2009 a new subset of T helper cells was discovered which is able to produce IL-22 but not IL-17 or IFNγ. This T cell subtype, distinct from Th1 and Th17 cells, was named Th22. Th22 cells share with Th17 cells some characteristics, such as the dependence on the master transcription factor RORC for the expression of their respective signature cytokines (Trifari et al., 2009). Moreover, Th22, like Th17 cells express the surface molecules CCR6 and CCR4. In addition to these surface markers Th22 cells also express CCR10, which allows for the migration of these cells to the epithelium, following a CCL27 gradient (Duhen et al., 2009; Trifari et al., 2009; Wang et al., 2010). Accordingly, Th22 cells are localized in the epidermis in inflammatory skin diseases (Eyerich et al., 2009; Eyerich and Zielinski, 2014). Although IL-9 production was initially associated with Th2 cells, recently a subset of Th cells producing copious amounts of this cytokine has been identified in Th9 cells (Veldhoen et al., 2008; Staudt et al., 2010). Th9 cell differentiation is induced by TGFβ and IL-4 and is dependent on the expression of the transcription factors PU.1 and the interferon regulatory factor-4 (IRF-4) (Ramming et al.,

2012; Kaplan, 2013; Kaplan et al., 2015). Finally, regulatory T cells (Tregs) present a stark difference compared to the other T cell subtypes. These cells are in fact devoted to the modulation of immune responses and maintenance of self-antigen tolerance, thereby avoiding autoimmune responses. There are two known types of Tregs: natural regulatory T cells (nTregs), which differentiate in the thymus, and induced regulatory T cells (iTregs), which develop from naïve CD4 T cells in the periphery in the presence of TGFβ and IL-6. Tregs are characterized by their high expression of the IL-2 receptor alpha-chain CD25. In fact they were discovered when CD4 T cells depleted of CD25 positive cells were transferred into nude mice and generated a strong autoimmune response. Those mice could be rescued by the transfer of CD25 positive cells, within a short time after inoculation of the CD25 negative cells. Hence, CD25 positive cells are necessary to modulate effector T cell activity (Sakaguchi et al., 1995; 2008). Tregs are also characterized by the expression of their master transcription factor forkhead box P3 (FOXP3), which is essential for both the development and the function of these cells (Fontenot et al., 2003). Tregs also express the surface proteins CTLA-4 and GITR, while CD127 is down regulated, and by their production of TGFβ (Read et al., 2000; Valzasina et al., 2005; Yi et al., 2006; Seddiki et al., 2006). Another important marker of Tregs is the surface protein GARP (Tran et al., 2009; Zhou et al., 2013). Tregs exert their suppressive activity on effector T cells in more than one way. Some of these suppressive mechanisms rely on direct contact with other cells. For example Tregs can bind to dendritic cells (DC) through a CTL-4-CD80/CD86 interaction which induces the DC to produce indoleamine 2,3-dioxygenase, an enzyme capable mediating cell cycle arrest in T conventional cells (Puccetti et al., 2007). Moreover Tregs are able to lyse target cells via the expression of perforin and granzymes A and B (Grossman et al., 2004). Other mechanisms of suppression do not involve cell-cell contact. As a matter of fact, Tregs are capable of secreting inhibitory cytokines like IL-35, IL-10 and TGFβ which directly impair T conventional cells proliferation (Collison et al., 2007). Furthermore, they possess the ability to consume IL-2, thereby depriving conventional T cells of this important component for their growth (Pandiyan et al., 2007).

1.3 Th17 cells

Th17 cells are a subtype of effector CD4 T cells specialized in the host defense against extracellular bacteria and fungi such as *Staphylococcus aureus* and *C. albicans* (Ma et al., 2008; Puel et al. 2011). Murine Th17 cells are generated, both *in vivo* and *in vitro* in response

to specific cytokines such IL-6 and TGFβ in mice (Bettelli et al., 2006 Nature). According to Cosmi et al., human Th17 cells seem to arise from a naïve CD4+CD161+ T cell precursor upon exposure to IL-1β and IL-23 (Cosmi et al., 2008). IL-1β is a major promoter of Th17 differentiation, in both mouse and man, which synergizes with IL-6 and IL-23 (Acosta-Rodriguez et al., 2007; Ghoreschi et al., 2010; Hirahara et al., 2010). IL-1 receptor (IL-1R) conditional knockouts show an impaired Th17 differentiation and reduced incidence of EAE (Sutton et al., 2006; Ben-Sasson et al., 2009; Hirahara et al., 2010). In this case, the only effect of TGFβ seems to be an inhibition of the differentiation of naïve CD4+CD161- T cells towards the Th1 phenotype, thereby only indirectly favoring the rise of the Th17 population (Santarlasci et al., 2009; Annunziato et al., 2012 Review). Moreover, other groups have demonstrated that while TGFβ was essential for Th17 differentiation in mouse, it actually had an inhibitory effect on the differentiation of human Th17 cells (Acosta-Rodriguez et al., 2007; Cosmi et al., 2008; Sallusto et al., 2012). However, it was later shown that while high doses of TGFβ are detrimental to human Th17 cell differentiation, low doses of TGFβ favor the rise of this subpopulation (Wilson et al., 2007; Yang et al., 2008; Sallusto et al., 2012). Several studies have demonstrated the importance of IL-1, and the dispensability of TGFβ, also in the development of murine Th17 cells (Chung et al., 2009; Ghoreschi et al., 2010; Annunziato et al., 2013). Developing Th17 cells produce IL-21, which also acts on the cell in an autocrine fashion to activate STAT3, a transcription factor essential for Th17 differentiation. IL-6, IL-21 and IL-23 determine the activation of the transcription factor STAT3 which plays an important role in the expression of the master transcription factor of Th17 cells, RAR-related orphan receptor gamma t (RORγt) to which RORC is the human ortholog (Ivanov et al., 2006; Yang et al., 2007; Mathur et al., 2007; Korn et al., 2009). It was in fact shown that conditional STAT3 knockout mice have impaired Th17 differentiation, while overexpression of a constitutively active STAT3 can increase IL-17 production (Yang et al., 2007; Harris et al., 2007; Korn et al., 2009). In human, Hyper IgE syndrome (HIES) patients, which carry a dominant negative mutation in STAT3 show an impaired Th17 development which leads to a susceptibility to bacterial and fungal infections (Holland et al., 2007; Minegischi et al., 2007; Milner et al., 2008; Ma et al., 2008; de Beaucoudrey et al., 2008). STAT3 is capable of directly binding not only IL-17A and IL-17F gene promoters, thereby regulating their expression, but also intergenic elements, as shown in ChIP-seq assay analysis (Durant et al., 2010). Furthermore, STAT3 directly regulates IL-21, IL-21R and IL23R genes (Ghoreschi et al., 2010; Hirahara et al., 2010), cooperating with RORγt in their transcription (Chen et al., 2006; Wei et al., 2007; Korn et al., 2009). Interestingly, STAT3 controls both the expression

of IL-23R and its downstream signaling (McGeachy et al., 2009). STAT3 also controls the expression of several transcription factors involved in Th17 differentiation such as RORγt, IRF4, and Batf. STAT3 binds RORγt and it has been demonstrated that a lack of STAT3 results in poor RORγt expression (Laurence et al., 2007; Yang et al., 2007; Mathur et al., 2007; Durant et al., 2010; Hirahara et al., 2010). However, overexpression of STAT3 in the absence of RORC also resulted in low IL-17 expression, indicating that STAT3 expression and function is necessary but not sufficient for IL-17 expression (Zhou et al., 2007). Commitment to the Th17 phenotype ultimately depends on RORγt expression, as it is sufficient to drive Th17 differentiation when overexpressed in mice, while its deficiency determined the absence of infiltrating Th17 cells in an EAE model (Ivanonv et al., 2006; Sallusto et al., 2012). RORC drives the production of the signature cytokines of Th17 cells, IL-17A and IL-17F, and it has been shown, through chromatin immunoprecipitation assays, that RORγt binds several sites in the IL-17 gene. RORC also promotes the expression of IL-23 receptor in a STAT3 dependent manner. Several murine studies claimed that IL-23 seems not to contribute to Th17 differentiation because murine naïve T cells lack the IL-23 receptor (IL-23R) (Ivanov et al., 2006; Zhou et al., 2007; Ichiyama et al., 2008; Hirahara et al., 2010). However, other studies based on human cells, like the previously cited work by Cosmi and colleagues, have demonstrated a fundamental role played by IL-23 signaling in driving Th17 differentiation (Wilson et al., 2007; Cosmi et al., 2008). Despite these contrasting results, it is well established that upon IL-23 binding, IL-23R signaling is essential for stabilization of the Th17 phenotype (Veldohen et al., 2006; Nurieva et al., 2007; Zhou et al., 2007; Korn et al., 2009). IL-23 is of particular interest as it is a heterodimer composed of a p19 and a p40 subunit, the latter of which it shares with IL-12 (Oppmann et al., 2000). The sharing of the p40 subunit between IL-23 and IL-12 has been exploited to block both Th17- and Th1-driven autoimmune responses, through the use of monoclonal antibodies such as Ustekinumab (Okamoto et al., 2015; Teng et al., 2015). The importance of IL-23 was first recognized in 2003 when the analysis of IL-23p19-deficient mice revealed that these mice were resistant to EAE and had very few IL-17 secreting cells (Cua et al., 2003). Furthermore, it was shown that IL-23 promotes IL-17 production in activated T cells which, when cultured in the presence of IL-23 and transferred in different mice, are able to determine the transfer of EAE and CIA (Affarwal et al., 2003; Murphy et al.,2003; Langrish et al., 2005; Korn et al., 2009). IL-17 belongs to the IL-17 family of cytokines, homodimeric peptides of between 35 and 52 kDa (Rouvier et al., 1993; Ouyang et al., 2008; Nistala et al., 2009). This family includes IL-17A (also known simply as IL-17), and IL-17B through –F. IL-17E (also known as IL-25) is not

produced by Th17 cells but rather by Th2 cells, where it might be involved in allergic responses (Fort et al., 2001). While other members of the IL-17 cytokine family are encoded by genes located on different chromosomes, IL-17A and IL-17F are syntenic and located on chromosome 1 and chromosome 6 in the mouse and human genome, respectively (Korn et al., 2009). Apart from Th17 cells, both cytokines are also produced by γ:δ T cells, NK cells, NKT cells, eosinophils and neutrophils (Molet et al., 2001; Starnes et al., 2001; Ferretti et al., 2003; Zhou et al., 2005; Lockhart et al., 2006; Liu et al., 2007; Korn et al., 2009). Studies based on the comparison of IL-17A and IL-17F knockout mice in the setting of various diseases have demonstrated that the two cytokines have largely overlapping functions (Yang et al., 2008; Korn et al., 2009). Both cytokines act on several different cell types where they induce the expression of several different cytokines and chemokines such as IL-1β, IL-6, G-CSF, GM-CSF, CXCL1, CXCL8, CXCL10, and matrix metalloproteinases (Yao et al., 1995; Fossiez et al., 1996; Janovic et al., 1998; Awane et al., 1999; Laan et al., 1999; Martel-Pelletier et al., 1999; Witowski et al., 2000; Hymowitz et al., 2001; Park et al., 2005). Both cytokines are essential for the recruitment, activation and migration of neutrophils (Korn et al., 2009). Th17 cells are also able to produce IL-6, IL-21, which are both able to enhance their own expression in an autocrine positive feedback loop, IL-22, IL-26, TNF β (Ouyang et al., 2008 Immunity). Another important IL-17-producing T cell subtype that has increasingly gathered attention in recent years is Th1/Th17. These cells, which will be the object of further discussion in another paragraph, are characterized by the co-production of IL-17 and IFNγ, and the expression of both Tbet and RORC. Another transcription factor important for induction of Th17 cells is the interferon regulatory factor 4 (IRF4). It was shown that IRF4 knockout mice were resistant to EAE and T cells and from these mice did not upregulate RORγt in the presence of TGFβ and IL-6, being in fact unable to differentiate into Th17 cells (Brustle et al., 2007). Beyond the factors cited above, there are several others that positively regulate Th17 cells, such as Batf, Runx1, NFAT, NF-κB, and also several negative regulators such as STAT1, IL-27, IL-2, STAT5, FOXP3 and SOCS3 (Hirahara et al., 2010). However, a detailed description of these mechanisms of positive and negative regulation of Th17 cells is beyond the scope of this thesis. Once in the site of inflammation Th17 cells secrete IL-8, which activates neutrophil granulocytes (Pelletier et al., 2010 Blood), and induce tissue resident cells, such as epithelial and endothelial cells, fibroblast and macrophages, to produce colony stimulating factor (CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and CXCL8, which recruits neutrophils to the site of inflammation (Ouyang et al., 2008 Immunity, Annunziato et al., 2013). IL-22 belongs to the IL-10 family of cytokines which is

produced by terminally differentiated Th17 cells in response to IL-23, and by Th22 cells which produce IL-22 but not IL-17 (McGeachy et al., 2007; Korn et al., 2009). IL-22 acts on receptors present in the skin, lung and gut to promote innate immune responses in these organs. Signal transduction through IL-22R determines the activation of MAPK pathways and activates, among others, STAT3 (Xie et al., 2000; Kotenko et al., 2001). Surprisingly, CCR6+ mononuclear cells, which include Th17 cells, have been shown to be capable of IL-10 production, an anti-inflammatory cytokine (Rivino et al., 2010). Furthermore, IL-10 production by these cells was shown to be upregulated by IL-23 and IL-27, while strongly and irreversibly inhibited by IL-1β, although overall IL-10 upregulation was limited to 3-5 days after stimulation (Zielinski et al., 2012; Sallusto et al., 2012). Another phenotypic characteristic of Th17 cells is their expression of a pattern of surface markers, additionally to CD161 cited above. In their work Acosta-Rodriguez et al. have found that all CCR6+ T cells were IL-17-producing and expressing RORC mRNA. They also found that among these cells, those co-expressing CCR4 represented the majority of antigen-specific memory T cells which, while proliferating, produced high amounts of IL-17 (Acosta-Rodriguez et al., 2007).

1.4 SGK1, FOXO1, IL-23R pathway

As discussed above, IL-23 signaling plays an important role in Th17 lineage commitment and stability and, consequently, it is involved in the pathogenesis of several autoimmune diseases. Moreover, the incidence of autoimmune diseases has increased in the last decades, indicating that changes in environmental factors, such as diet, might play a role in the rise of these diseases. Salt consumption has increased steadily in the Wester diet with the increased consumption of processed foods. Recently, this environmental risk factor has been linked to autoimmune diseases (Farez et al., 2014; Sundstorm et al., 2014; Binger et al., 2015). Two publications in particular have shed light on the effects of high salt intake in Th17 cells development and in autoimmune disease models. Kleinewietfeld et al. (2013) have shown a significant increase in the expression of Th17 signature cytokines upon culture with a concentration of 40mM NaCl in the culture medium. This corresponds to the NaCl concentration in the interstitium of mice on a high salt diet. These high concentrations of NaCl during Th17 polarization resulted in the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and in an increased expression of the osmosensitive transcription factor nuclear factor of activated T cells 5 (NFAT5), and it's target serum/glucocorticoid-regulated kinase1 (SGK1) (Shapiro and Dinarello., 1995; Aramburu and López-Rodríguez, 2009; Kleinewietfeld et al., 2013; Binger et al., 2015). Moreover, mice fed with high

quantities of salt developed a more severe form of experimental autoimmune encephalomyelitis (EAE) and had a higher frequency of Th17 cells, compared to mice with a normal diet (Kleinewietfeld et al., 2013).

Wu et al. (2013) have demonstrated that IL-17-producing CD4 T cells lacking a functional Sgk1 through conditional knockout mice, specifically $Il17f^{Cre}Sgk1^{flox/flox}$ mice, could not maintain a stable Th17 phenotype when cultured with IL-23. Moreover, the severity of EAE was milder in knockout mice. Activated SGK1 is able to phosphorylate the transcription factor forkhead box protein O1 (FOXO1). FOXO1 elicits its functions in the nucleus. It is in particular capable of suppressing Il23r expression by actively binding the gene, therefore impeding RORγt binding which is essential for promoting expression of this gene. Upon FOXO1 phosphorylation by SGK1, phospho-FOXO1 translocates to the cytoplasm, thereby freeing the Il23r gene for RORγt binding. Lastly, the authors demonstrated a potentiated Th17 differentiation and an increased stability of the phenotype, both in vitro and in vivo, as well as an augmented severity of EAE in response to increased NaCl concentrations (Wu et al., 2013). As discussed above, SGK1 activity is essential to IL-23R expression. However, IL-23 signaling was shown to induce and maintain SGK1 expression in Th17 cells, thereby constituting a positive feedback loop, which helps to maintain and stabilize the Th17 phenotype. This model is in accordance with the fact that SGK1 is not required for Th17 differentiation (Heikamp et al., 2014; Norton and Screaton., 2014).

1.5 Th17 cells in rheumatoid arthritis and other autoimmune diseases

Rheumatoid arthritis (RA) is a systemic autoimmune diseases affecting 0.5-1% of the population of developed countries, with about three fold incidence in women. RA is a highly invalidating disease which results in a lower quality of life, disability, and reduced life expectancy (Alamanos and Drosos, 2005; Mellado et al., 2015). RA is characterized by chronic inflammation of synovial tissues, which leads to cartilage and bone destruction. Other organs are also affected and can become inflamed to the point where there can be cardiovascular, pulmonary and skeletal complications (McInnes et al., 2011). The origin of the events that lead to initiation of the disease are not yet clear. RA is a polygenic disease that involves genetic, epigenetic and environmental factors. This disease is characterized by changes in the synovium, with hyperplasia, neoangiogenesis and local infiltration by immune cells, particularly T cells, as evidenced by T-cell dependent arthritis in mouse models and by the development of drugs that target T cell co-stimulation, such as *abatacept* (Buch et al., 2009; Hot and Miossec, 2011). Among the infiltrating T cells, Th17 cells have a prominent

role in the pathogenesis of rheumatoid arthritis. First, IL-17 was found to be elevated in the joints of RA patients compared to the peripheral blood of the same RA patients or to the synovial fluid (SF) from patients with osteoarthritis (OA) (Kotake et al., 1999). It was later demonstrated that both the levels of IL-17 and the frequencies of Th17 cells in the peripheral blood of RA and psoriatic arthritis (PsA) patients strongly correlated with DAS28 and levels of C-reactive protein (CRP) (Leipe et al., 2010). IL-17 binds type 1 transmembrane protein receptors, which can have a homodimeric or heterodimeric configuration, the latter of which is composed of IL-17RA and RC subunits (Nistala and Wedderburn, 2009). These receptors are found among different cell types such as B- and T lymphocytes, synovial fibroblasts and chondrocytes (Honorati et al., 2001; Zrioual et al., 2008; Nistala and Wedderburn, 2009). In RA, IL-17 contributes to cartilage and bone destruction in several different ways (Chabaud et al., 2001; Nistala and Wedderburn, 2009). IL-17 induces the release of matrix metalloproteinases from synovial fibroblasts, which dismantle cartilage (Koshy et al., 2002). In synergy with IL-1 and TNFα, IL-17 induces the release of pro-inflammatory cytokines from monocytes and synovial fibroblasts, including IL-6, and IL-1 and TNFα themselves. Also IL-8 and GM-CSF are released, both of which stimulate neutrophil recruitment to the joint. However, once the arthritis is established, IL-17 can maintain the disease in the absence of TNFα (Chabaud et al., 1998; Jovanovic et al., 1998; Katz et al., 2001; Koenders et al., 2006; Nistala and Wedderburn, 2009). Finally, IL-17 and IL-1 induce the expression by osteoblasts of receptor activator of nuclear factor-κB ligand (RANKL), which enhances the maturation of osteoclasts, ultimately resulting in bone destruction (Lubberts et al., 2003). Apart from Th17 cells, Treg malfunction plays a fundamental role in the pathogenesis of RA. As mentioned in the previous chapter, regulatory T cells play an important role in controlling inflammation through various mechanisms. In RA, TNFα contrasts the suppressive function of Tregs on effector T cells by hindering Foxp3 mRNA expression. Anti-TNFα angents (TNFi) block such TNFα-mediated functions (den Broeder et al., 2002; Ehrenstein et al., 2004). TNF inhibitors have demonstrated very high efficacy and are therefore widely used. However, a lack of response to anti-TNFα drugs has been observed in patients with high baseline levels of Th17 cells (Alzabin et al., 2012). Furthermore, anti-TNFα therapy is associated with an increased risk of reactivation of tuberculosis (Dixon et al., 2010; Mewar and Wilson, 2011). Alone or in conjunction with anti-TNFα, conventional disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate are widely used (Singh et al., 2012; Kunwar et al., 2016). However, remission is achieved only in 30-40% of the patients treated with DMARDs only (Storage et al., 2010; Kunwar et al., 2016). Hence, there is a need to

explore different therapeutic options which involve new biologics. *Secukinumab* and *ixekizumab* are both MAb, targeting IL-17A, currently in phase III and completed phase II clinical trials for RA, respectively (Kugyelka et al., 2016). *Secukinumab* has been already approved for the treatment of psoriasis, psoriatic arthritis (PsA) and in spondyloarthritis. Previously, the phase II trials revealed clinically relevant response in RA patients unresponsive to treatment with conventional DMARDs or other biologics (Genovese et al., 2014). *Ixekizumab* is also a recently FDA approved drug for the treatment of psoriasis, and PsA. A recent phase III study revealed that *ixekizumab* was well tolerated in RA patients, who showed clinical improvements. These positive effects of the drug were maintained or improved through week 64 (Genovese et al., 2016). *Brodalumab* is a MAb which targets IL-17RA. However, *brodalumab* seems to be ineffective in RA (Pavelka et al., 2015). In conclusion, so far anti-IL-17 therapy has led to mixed results and overall did not prove to be a therapy preferable to current therapeutic strategies for the treatment of RA. However, more studies are needed to further explore the possibility of using anti-IL-17 strategies, particularly in those cases where other therapeutic strategies conveyed unsatisfactory results.

1.6 Plasticity

The Th1/Th2 paradigm first proposed by Mosmann, Coffman and colleagues in the mid-80s had the prerogative of considering polarized lineages mutually exclusive and stable, due to the self-enforcing feedback loops essential to the acquisition of the phenotype these T cell subsets (Mosmann et al, 1986; Mosmann and Coffman, 1989; Heinzel et al., 1989; Muranski and Restifo, 2013). This view of terminal differentiation was also initially applied to newly discovered T cell subtypes such as Tregs and Th17 cells. However, IL-17/IFNγ double positive cells (Th17/Th1) were soon found *in vivo* under pathological conditions such as Crohn's disease, demonstrating that the cells could at least acquire this mixed phenotype (Annunziato et al., 2007). The notion that Th17 cells could undergo a late developmental plasticity was analyzed in detail using IL-17F reporter mice. In this study by Lee and colleagues, Th17 cells maintained a stable phenotype when cultured in the presence of TGFβ and IL-23 but rapidly shifted to IFNγ producing cells upon re-stimulation in the presence of IL-12 or IL-23 and in the absence of TGFβ. This shift occurred also *in vivo* where cells expressing IL-17F become IFNγ-producing colitogenic effector cells (Lee et al., 2009). Also the group of Romagnani and colleagues has proven that *in vitro* Th17 clones can acquire IFNγ production in addition to IL-17A (Annunziato et al., 2007). Furthermore, they have shown

high frequencies of CD4 T cells positive for CD161, and secreting both IL-17A and IFNγ, in the synovial fluid (SF) of juvenile idiopathic arthritis (JIA) patients. Moreover, when Th17 cells were isolated from the SF of these patients and cultured in medium alone, they spontaneously shifted to an IFNγ-producing phenotype. Also Th17 cells isolated from the peripheral blood (PB) of healthy individuals underwent the same shift when cultured in the presence of IL-12 or the synovial fluid of JIA patients (Cosmi et al., 2011). As mentioned in the previous paragraphs, IFNγ-positive IL-17-negative cells that are also positive for CD161 are called "non-classic" Th1 cells and are generated through plasticity of Th17 cells. Although these cells do not produce IL-17, they express RORC as well as Tbet, CCR6, and IL-23R (Maggi et al., 2012). On the other hand, "classic" Th1 cells are CD161 negative and arise from naïve CD4 T cells (Maggi et al., 2012; Annunziato et al., 2013). In a study by a different group it was also found that these ex-Th17, non-classic Th1 cells from the synovial fluid of JIA patients, co-express IFNγ and GM-CSF (Piper et al., 2014). Additionally, a recent study found an inverse correlation between the frequency of non-classic Th1 cells in the peripheral blood of early-onset RA patients and the titer of anti-CCP antibodies in RA, suggesting for a more important role played by Th17 cells in the pathogenesis of early phase RA (Kotake et al., 2016). Th17/Th1 cells were found not only in the joints of JIA patients, but also in the gut of inflammatory bowel disease (IBD) patients and in the central nervous system (CNS) of multiple sclerosis (MS) patients. These cells were shown to cross the blood-brain barrier and home to the CNS in experimental autoimmune encefalomielytis (EAE) mice, a model for human MS (Annunziato et al., 2007; Kebir et al., 2009; Geginat et al., 2014). Th17/Th2 cells, capable of producing both IL-17 and IL-4, have also been found in the PB of healthy individuals and, in a significantly higher percentage, in the PB of asthmatic patients. In the same study it was also shown that CCR6+CD161+CD4+ cells, which contain high frequencies of Th17 cells, readily shifted towards a Th17/Th2 phenotype upon culture in the presence of IL-4 (Cosmi et al., 2010).

Th1 cells are widely considered to be more stable in their phenotype, compared to Th17 cells. However, already in a study from 2003, Th1 cells could be repolarized to acquire a Th2 phenotype upon IL-4 stimulation (Messi et al., 2003). In a more recent study, plasticity of Th1 cells towards a Th2 phenotype following helminth infection has been reported (Panzer et al., 2012). Furthermore, Th1 cells sorted from IFNγ reporter mice are able to acquire a Th17-like phenotype based on the increased RORγt and decreased T-bet expression and Th1 cells readily convert to both Th17 and Th1/Th17 cells in the inflamed colon of mice (Brown et al., 2015; Liu et al., 2015).

Similarly to Th1- also Th2 cells are also considered to be rather stable. However, plasticity of murine Th2 cells *in vitro* and *in vivo,* whereby they adopted a mixed Th2/Th1 phenotype, has been demonstrated (Hegazy et al., 2010).

1.7 Epigenetic modifications of the histones and plasticity

Differentiated cells have the potential to maintain their phenotype over several rounds of replication. However, as above stated, several CD4 T cell types are able to plastically change their phenotype in terms of cytokine production pattern. One important aspect governing lineage stability and, therefore, plasticity of CD4 T cells is their epigenetic configuration. The term epigenetics refers to underlying mechanisms that preserve cellular memory and maintain distinctive transcriptional profiles and cellular identity (Kanno et al., 2013). Epigenetic modifications include DNA methylation and modifications of the histone tails. DNA methylation consists of the modification of cytosines in certain CpG dinucletides through the addition, by a methyltransferase, of a methyl group to the fifth carbon of said cytosine residue. Such modification can be associated with transcriptional repression when it occurs at gene promoters. This transcriptional repression can be caused either by a direct interference of the methyl group with the binding of transcription factors to the promoter region of a gene, or by the recruitment of methyl-CpG-binding domain (MBD) protein family members with chromatin remodeling activities. Therefore, MBD proteins represent a link between histone modifications and DNA methylation (Hashimoto et al. 2010). DNA methylation has been shown to have a role in the plasticity of Th cell subtypes (Thomas et al., 2012). However, a detailed description of this phenomenon is beyond the scope of this thesis.

Another important form of epigenetic modifications are those that influence the structure and condensation level of chromatin. A tightly packed chromatin provides, in fact, a physical barrier which can constitute an obstacle to gene transcription (Roeder, 2005). The functional chromatin unit which can be compacted or released is the nucleosome. The nucleosome is a cylindrical protein complex constituted by an octamer of four histones: H2A, H2B, H3, H4 and the linker H1. Around this protein complex, double stranded DNA 146 base pairs in length, is coiled. Furthermore, histones can be modified through catalytic enzymes able to add or remove methyl, acetyl, phosphate, ubiquitin, sumoyl and ADP-ribose groups to different amino acids located at the N-terminal histone tails (Kouzarides, 2007). The modification of the histone tails determine condensation or relaxation of chromatin, thereby allowing or denying access to regulatory regions of the genes by transcription factors (Grunstein, 1997). Acetylation alters histone charge displacing them from DNA, thereby making regulatory

sequences more accessible to transcription factors, enhancing transcription (Bannister and Kouzarides, 2011). This type of modifications are called “permissive”, while modifications that determine a condensation of the chromatin are classified as “repressive”. While acetylation is prerogatively permissive, methylation can be either permissive or repressive depending on the position and type of amino acid residue that is modified. For example trimethylation of lysine 4 at histone H3 (H3K4me3) is associated with a permissive mark, while trimethylation of lysine 27 at histone 3 (H3K27me3) is associated with a repression of transcription and is often found at regions with a closed chromatin configuration (Cervoni et al. 2001; Ooi et al. 2007; Wei et al., 2009).

In humans there are several methyltransferases which are specifically active at lysine 4 of histone 3 (H3K4). They all belong to the mixed-lineage leukemia (MLL) family of genes, which comprise SET1A, SET1B, MLL1, MLL2, and MLL4. The first member of this family to be discovered, MLL1, is associated with chromosomal translocations in some aggressive leukemias, hence the name (Ruthenburg et al., 2007). Furthermore, MLL1, in addition to its role in determining H3K4 methylation, associates with histone acetyltransferases, allowing for active gene transcription of specific regions (Milne et al. 2002; Zhao et al. 2013).

Histone modifications play an important role in CD4 T cell lineage determination and stability (Agarwal and Rao, 1998). Genome-wide ChIP-seq experiments by Wei and collegues (2009) have shown that epigenetic modifications associated with cytokine genes such as IFNγ, IL-4 and IL-17 were coherent with what expected in differentiated Th1, Th2 and Th17 cells, respectively, with permissive H3K4me3 marks for each gene in the corresponding T cell lineage and repressive H3K27me3 in genes that are not characteristic for the specific lineage. However, some of the master transcription factors of these cell lineages have shown a different pattern of histone modifications which included, for example, permissive H3K4me3 at both the RORC and Foxp3 loci in iTregs, with no repressive H3K27me3 marks. Moreover, the master transcription factor TBX21 was characterized by both permissive and repressive modifications in cells differentiated under all conditions (Wei et al., 2009). This bivalent configuration of opposing histone modifications has been previously associated with genes poised for expression, a characteristic which might facilitate the production of IFNγ by non-Th1 cells, in the context of a plastic shift towards a Th17/Th1 phenotype (Azuara et al., 2006; Bernstein et al., 2006). On the other hand, the fact that the Il17 locus is repressed in non-Th17-polarized cells, might indicate a resistance of these cells towards acquiring the mixed Th17/Th1 phenotype (Wei et al., 2009). Furthermore, Akimzhanov and colleagues (2007) also reported that Th17 cells show increased permissive

H3 acetylation at the Il17a and Il17f gene promoters and at several conserved non-coding sequences (CNSs) belonging to the same locus, compared Th1 and Th2 cells (Akimzhanov et al., 2007). Regarding the role of epigenetic modifications in the plasticity phenomenon, important contributions were given by Mukasa and colleagues in 2010 and by Cohen and colleagues in 2011, using mouse and human cells, respectively. In the study by Mukasa and colleagues, mouse Th17 cells were analyzed for histone modification at the Ifng and Il17a and Il17f loci in isolated Th1, Th17 cells, and cells cultured either in the presence of TGFβ or IL-12 in order to maintain the Th17 phenotype or induce a plastic shift towards the Th1 phenotype. The analysis of histone modifications at the Ifng promoter and various CNS regions revealed, as expected, a mostly permissive pattern of H3K4me3 in isolated Th1 cells, while in Th17 cells the epigenetic marks where mostly either repressive (H3K27me3) or "neutral". However, both the Th17 cells cultured in the presence of IL-12 and those cultured in the presence of TGFβ, showed a mostly permissive pattern of histone modifications for all regions, with the exception of CNS-54, but particularly in the cells cultured in the presence of IL-12. At the Il17 locus there was, instead, a clear prevalence of permissive modifications in the Th17 cells cultured in the presence of TGFβ, while those cultured in the presence of IL-12 showed a clearly dominant pattern of repressive epigenetic marks. The authors, further proved that the change in epigenetic landscape at the Il17 gene locus was dependent on Rorc, whose epigenetic status was repressed by IL-12 signaling through STAT4 (Mukasa et al., 2010). The works cited so far used cells derived from animal models to identify the epigenetic status of T cell subtypes and plasticity phenomena. However, very few publications have used human cells to address these topics. One such publication was published by Cohen and colleagues in 2011. In this study the authors showed that upon transdifferentiation of isolated Th17 cells under Th1 inducing conditions, the Ifng promoter acquired more permissive H3K4me3 modifications compared to Th17 cells cultured under neutral conditions, although this difference was not statistically significant. Moreover, at the Il17a gene promoter, the cells cultured under Th1 inducing conditions maintained permissive modifications to the same extent as cells cultured under neutral conditions, but acquired significantly higher levels of the repressive H3K27me3 mark. However, the histone modifications in the setting of Th17 plasticity towards a Th1 phenotype proved to be rather modest. Furthermore, also isolated Th1 cells cultured under Th17 inducing conditions did not induce significant changes in the histone methylations pattern at the Ifng and Il17a gene promoters as well as at TBX21, and RORC2 gene promoters. The authors concluded that although Th1, and especially Th17 cells, can transiently acquire a mixed Th1/Th17 phenotype in response to Th17 or Th1 inducing

conditions, respectively, this phenotypic change does not fully reflect changes at the epigenetic level (Cohen et al., 2011).

A very important and recent study was conducted by Mazzoni and colleagues in 2015. In this study the authors analyzed the methylation status of the IL17A, IL17F and IFNG promoters, and regions of interest within RORC2 and TBX21 gene loci in either clones or freshly sorted Th17, Th17/Th1 and classic and non-classic Th1 cells. They found a resemblance of the DNA methylation status between non-classic Th1 cells and Th17 or Th17/Th1 cells, particularly with regard to the methylation status of the RORC2 downstream promoter, which was completely demethylated in Th17, Th17/Th1 and non-classic Th1 cells, while it was completely methylated in classic Th1 cells. Furthermore, demethylation of the IFNG promoter accompanied transidifferentiation of Th17 cells towards the Th17/Th1 and Th1 phenotype. These findings support the origin of non-classic Th1 from Th17 cells as shown by their demethylation of RORC2 and IL17A regions of interest and, although it is not yet clear if as a cause or an effect, argue in favor of a role for DNA methylation in T cell plasticity (Mazzoni et al., 2015).

1.8 Aim of the thesis

Th17 cells have been demonstrated to be among the major contributors to the pathogenesis of autoimmune disease and, among these, rheumatoid arthritis. These cells have been previously shown to be in increased frequencies in the PB and SF of RA patients. Plastic phenomena have been shown in both in vitro and in vivo studies. However, plasticity of T helper cells from and towards the Th17 phenotype in the context of rheumatoid arthritis has not yet been fully pursued. The aim of this thesis therefore is:

- To investigate a possible role for CD4 T cell plasticity in the observed increased frequency of Th17 cell in the peripheral blood of well defined cohort of patients with early RA, compared to HC, using *in vitro*- or in *vivo*-generated CD4 T cell subsets.
- To identify possible variations in the expression levels of key cytokine and transcription factor loci, as well as genes involved in Th17 lineage stability, in CD4 T cells from RA patients compared to healthy controls.
- To investigate the epigenetic status, in terms of histone modifications, at the master transcription factor loci of *in vivo*-generated Th1 and Th17 cells derived from RA and HC.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Chemical / Reagent	Manufacturer
Agarose	Merck
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, St. Louis, MO, USA
β-mercaptoethanol	Sigma-Aldrich
Bovine serum albumin (BSA)	Merck
Dimethylsulfoxid (DMSO)	Merck
Dithiothreitol (DTT)	Sigma-Aldrich
DNA Gel Loading dye (6x)	Life technologies
dNTP set (100 mM solutions)	Life technologies
Ethanol (C_2H_5OH)	Merck
Ethylendinitrotetraacetic acid (EDTA)	Sigma-Aldrich
Ficoll lymphoflot	Biotest, Dreieich, Germany
Formaldehyde 37% (CH_2O)	AppliChem GmbH, Darmstadt, Germany
L-glutamine ($C_5H_{10}N_2O_3$)	Life technologies
Glycine (NH_2CH_2COOH)	Merck
Hanks' Balanced Salt Solution (HBSS) with phenol red	Thermo Scientific
Heparin-sodium salt	Ratiopharm, Ulm, Germany
4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid (HEPES)	Merck
Hydrochloric acid 37% (HCl)	Merck
Ionomycin	Merck
Magnesium chloride ($MgCl_2$)	Merck
Monensin	Sigma-Aldrich
NP-40	Millipore, Billerica, MA, USA
Oligonucleotide-dT_{12-18} (Oligo(dT))	GE Healthcare
Paraformaldehyde (PFA)	Merck
Penicillin G/streptomycin	Life technologies
Phenylmethylsulfonylfluorid (PMSF) ($C_7H_7O_2SF$)	Roche, Penzberg, Germany
Phorbol myristate acetate (PMA)	Sigma-Aldrich

Phosphate buffered saline (PBS)	Life technologies
Piperazine-N,N‘-bis-2-ethanesulfonicacid (PIPES)	Millipore
Potassium chloride (KCl)	Merck
Power SYBR® Green PCR Master Mix	Applied Biosystem
Protease Inhibitor Cocktail Tablets (complete, EDTA-free)	Roche
Roswell Park Memorial Institute (RPMI)1640	Life technologies
Saponin	Sigma-Aldrich
Sheep erythrocytes	Fiebig-Nährstofftechnik, Idstein, Germany
Sodium acetate (CH_3COONa)	Merck
Sodium azide (NaN_3)	Merck
Sodium chloride (NaCl)	Merck
Sodium deoxycholat ($C_{24}H_{39}NaO_4$)	Merck
Sodium dodecyl sulfate (SDS)	Merck
Sodium hydroxide (NaOH)	Merck
SYBR safe DNA gel stain (10,000x)	Life technologies
TaqMan Universal PCR mastermix 2x	Life technologies
TGF-β (recombinant human)	R&D Systems, Minneapolis, MN, USA
Tris(hydroxymethyl)-aminomethan (TRIS) ($C_4H_{11}NO_3$)	Merck
Triton X-100	Sigma-Aldrich
Tween 20	Sigma-Aldrich

2.1.2 Cytokines

Cytokine	***Final concentaration in culture***	***Provider***
Recombinant human IL-1ß	10 ηg/ml	R&D Systems
Recombinant human IL-2 (Proleukin)	10 U/ml	Novartis, Basel, Switzerland
Recombinant human IL-4	31,25 ηg/ml	Perbio Science, Bonn, Germany
Recombinant human IL-12	40 ηg/ml	R&D Systems
Recombinant human IL-21	100 ηg/ml	Invitrogen
Recombinant human IL-23	20 ηg/ml	R&D Systems

Recombinant human TGFß	5 ηg/ml	R&D Systems

2.1.3 Antibodies

Specificity	***Conjugate***	***Clone***	***Provider***
Antibodies for cell culture			
Anti-human CD3	non	OKT3	LGC Standards Teddington, UK
Anti-human CD28	non	CD28.2	BD Biosciences, San Diego, CA, USA
Anti-human IL-4	non	25D2	Thermo Scientific
Anti-human IFNγ	non	Polyclonal	Thermo Scientific

Specificity	***Conjugate***	***Clone***	***Provider***
Antibodies for flow cytometry (surface staining)			
Anti-human CD3/CD4 dual tag	FITC/PE	UCHT1/Q4120	BD Biosciences
Anti-human CD45RA	FITC	HI100	BD Biosciences
Anti-human CD45RO	PE	UCHL1	BD Biosciences
Anti-human CD27	PE	M-T271	BD Biosciences
Anti-human CD27	FITC	M-T271	BD Biosciences

Specificity	***Conjugate***	***Clone***	***Provider***
Antibodies for flow cytometry (intracellular staining)			
Anti-human IL-4	APC		BD Biosciences
Anti-human IL-9	PE	MH9A4	BioLegend
Anti-human IL-17	PE/Cy7	BL168	BioLegend
Anti-human IFNγ	FITC	4S.B3	BD Biosciences

Specificity	***Clone***	***Provider***
Antibodies for Chromatin Immunoprecipitation (ChIP)		
Anti-trimethyl-Histone H3 (Lysine4)	polyclonal	Merck

anti-acetyl-Histone H3	polyclonal	Merck
anti-trimethyl-Histone H3 (Lysine27)	polyclonal	Merck
Normal rabbit IgG (isotype control)	polyclonal	Cell Signaling

2.1.4 Ladders/Markers

Name	***Provider***
GeneRuler DNA ladder (100 bp)	Fermentas, St. Leon-Rot, Germany
GeneRuler DNA ladder (1 kb)	Fermentas

2.1.5 Serum

Name	***Provider***
Fetal calf serum (FCS)	Life technologies
Mouse serum	Sigma-Aldrich
Normal human serum (NHS)	From our lab, Munich, Germany
Rat serum	Sigma-Aldrich

2.1.6 Enzymes

Enzyme	***Supplied Reaction Buffer***	***Provider***
AmpliTaq DNA polymerase	10x PCR Buffer II	Life technologies
Epimark Hot Start Taq DNA Polymerase	5x Epimark Hot Start Taq reaction buffer	New England Biolabs
GeneAmp High Fidelity PCR Enzyme Mix	10x GeneAmp High Fidelity PCR buffer	Life technologies
peqGOLD Proteinase K (20 mg/ml)		Peqlab
RNase A, DNase and protease-free (10 mg/ml)		Life technologies

2.1.7 TaqMan Gene Expression assays

Gene	*Assay ID*	*Probe*	*Sequence (5‘ to 3‘)*
Cyclophilin A	4310x10783E	VIC-probe	not provided
b-actin	4310x10781E	VIC-probe	not provided
IFNG	Hs00989291_m1	FAM-probe	not provided
IL-4	Hs00174122_m1	FAM-probe	not provided
IL-17	Hs00176383_m1	FAM-probe	not provided
TBX21	Hs00203436_m1	FAM-probe	not provided
GATA3	Hs00231122_m1	FAM-probe	not provided
RORC	Hs01076112_m1	FAM-probe	not provided

For - Forward; Rev - Reverse

2.1.8 Oligonucleotides

Gene/Region	*Oligonucleotide*	*Sequence (5‘ to 3‘)*
ChIP assay realtime-polymerase chain reaction (PCR) primer		
RORC Promotor	For primer	TCT CCC CTA TGC CTG TCA CCT G
	Rev primer	TGA TTT TGC CCA AGG ACT CAC AC
TBX21 Promotor	For primer	GGC AAC CCG AAA GGT CAC TTA G
	Rev primer	TTC TCC TGG CAC TCA GAG GCT C

For - Forward; Rev – Reverse

2.1.9 Instrumentation

Instrument	*Manufacturer*
ABI Prism 7000 Sequence Detection system	Life technologies
Biological Safety Cabinets NU-437 Class II, Type A2	Nuaire
BioPhotometer	Eppendorf, Hamburg, Germany
Bioruptor® Plus (B01020001)	Diagenode, Denville, NJ, USA
Cell counter Z1, Z2	Beckman Coulter
Centrifuge 5417R, 5415D, 5415R, 5430	Eppendorf
FACSAria™ III	BD Biosciences
FACS Cytomics FC500	Beckman Coulter

FACS MoFlo Legacy	Beckman Coulter
FUJIFILM LAS-3000	Fujifilm, Tokyo, Japan
Gel chamber Nautico 10x107	H. Hölzel Laborgeräte GmbH, Dorfen, Germany
HERAcell 240 CO2 incubator	Thermo Scientific
MACSmix Tube rotator	Miltenyi Biotec
MACS® Separators	Miltenyi Biotec
Microcentrifuge 5415R	Eppendorf
pH meter In Lab Routine Pro	Mettler-Toledo
Reax 2000 Vortex mixer	Heidolph
Rotixa 50 RS centrifuge	Hettich AG, Bäch, Switzerland
Thermomixer comfort	Eppendorf
UVT-28 MP transilluminator	Herolab GmbH, Wiesloch, Germany
Water bath GFL 1083	GFL Gesellschaft für Labortechnik GmbH

2.1.10 Kits

Kit name	***Origin***
Affinity Script QPCR cDNA Synthesis kit	Agilent technologies
ChIP-IT High Sensitivity® (HS) Kit, 16 rxns	Active Motif
Memory CD4+ T Cell Isolation kit, human	Miltenyi Biotec
Naïve CD4+ T Cell Isolation kit II, human	Miltenyi Biotec
QIAamp DNA Blood Mini kit	Qiagen, Hilden, Germany
RNeasy Plus Mini kit	Qiagen

2.1.11 Buffers and solutions

Buffers and solutions	***Composition***
0,5 M EDTA, pH 8,0	
0,1 M NaOH	
1 M TrisHCL, pH 9,5	
1 M TrisHCL, pH 8,0	
10 % NaN_3	
5 % Saponin	5 % Saponin in PBS
Cell culture medium	500 ml RPMI 1640 2 mM L-Glutamine 50 U/ml Penicillin G

	50 µg/ml Streptomycin 10% NHS
Cell lysis buffer	5 mM PIPES, pH 8.0 85 mM KCl 0.5% NP-40 1x PIC
6x DNA loading buffer	50 mM EDTA 26.1% glycerol 0.25% bromphenol blue
FACS buffer	PBS 2% FCS 0.01% NaN_3
FACS-Saponin	2% Saponin solution in FACS buffer
MACS buffer	PBS 0.5% BSA 2 mM EDTA
10x NH_4Cl	41.45 g NH_4Cl 5 g $KHCO_3$ 1 mM EDTA H_2O ad 0.5 l
Nuclear lysis buffer	50 mM Tris-HCl, pH 8.0 10 mM EDTA 0.1% SDS 1x PIC
Sonication buffer	50 mM HEPES, pH 7.8 140 mM NaCl 1 mM EDTA 1% Triton X-100 0.1% Na-Deoxycholat 0.1% SDS 0.5 mM PMSF 1x PIC
Swelling buffer	25 mM HEPES, pH 7.8 1.5 mM $MgCl_2$ 10 mM KCl 0.1% NP-40 1 mM DTT 0.5 mM PMSF 1x PIC
50x Tris-Acetate-EDTA (TAE) buff**er**	242 g Tris-HCl 57.2 ml acetic acid 50 mM EDTA (pH7.6) H_2O ad 1 l
TBST	20 mM Tris-HCl (pH7.6) 140 mM NaCl

	0.1% Tween-20
TE buffer	10 mM Tris-HCl, pH 8.0 1 mM EDTA 0.5 mM PMSF 1x PIC

2.1.12 Software

Software	***Developer***
Corel Draw, Version 11.633	Corel Corporation
GraphPad Prism, Version 5.0a	GraphPad Software
MacVector Software	MacVector, Inc., Cary, NC, USA
Microsoft Excel, Version 11.4.1	Microsoft Corporation
Vista Genome Browser	Genomics Division of Lawrence Berkeley National Laboratory

2.2 Methods

2.2.1 Study population

Peripheral blood (PB) was obtained at the time of first clinical evaluation from 58 patients (Table 1) who fulfilled the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 criteria (Aletaha et al., 2010). The patients had active disease as defined by a Disease Activity Score in 28 joints (DAS28) of ≥ 3.2 and had a mean duration of less than 6 months since their initial clinical symptoms. In order to exclude any influence of drug administration on plasticity phenomena or epigenetic modifications, only blood from treatment-naive patients was used. Therefore, patients had never been treated with glucocorticoids or disease modifying antirheumatic drugs (DMARDs). Demographic and clinical parameters such as age, gender, smoking, disease duration, tender joint count (TJC), swollen joint count (SJC), DAS28 values, erythrocyte sedimentation rate (ESR) and levels of C-reactive protein (CRP), rheumatoid factor (RF), and anti-cyclic citrulinated peptide (anti-CCP) antibodies were collected at the time of blood sampling. For control, age-matched healthy individuals were analyzed (n=35) (Table 1). Written informed consent was provided by all patients and healthy donors. The study was approved by the ethics committee of the University of Munich.

Study population

Parameters	Healthy controls (n=34)	Patients with RA (n=58)
Age (years)	50.6 ± 8.2	55.4 ± 16.1
Female:male ratio (n)	25:7	41:17
Disease duration (months)	n.a.[a]	3.6 ± 2.7
RF positive (%)	n.d.[b]	79.3
Anti-CCP positive (%)	n.d.	60.3
DAS28	n.a.	5.0 ± 1.2
TJC (n)	n.a.	8.7 ± 6.9
SJC (n)	n.a.	7.2 ± 4.9
CRP (mg/dl)	n.d.	2.3 ± 3.1
ESR (mm/h)	n.d.	27.6 ± 26.0

data are shown as mean ± SD or absolute numbers
[a] n.a. not applicable
[b] n.d. not determined
anti-CCP, antibodies to citrullinated peptides; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count.

2.2.2 Cell purification

2.2.2.1 Isolation of human rosette-positive and -negative cells

In this study, we used isolated human CD4 T cells from healthy blood donors and rheumatoid arthritis patients (RA). As a first step PBMCs were isolated from peripheral blood through density gradient centrifugation. Briefly, 20 ml of heparinized blood per Falcon tube were diluted 1:2 by adding 20 ml of PBS, to a final volume of 40 ml. 10 ml of Ficoll (Biotest) were then layered at the bottom of the tube. The tubes were then centrifuged for 20 minutes, 400 g, at room temperature. After centrifugation four layers were clearly visible: an upper, yellow layer, composed of plasma and containing platelets, a thin white layer of mono-nuclear cells, the Ficoll layer and a thick red layer constituted of erythrocytes and granulocytes. PBMCs were harvested by suction with a 10 ml pipette, washed with PBS by centrifugation and counted with a Z2 Coulter counter. 0.01x106 cells were collected for successive surface molecule analysis and purity evaluation.

Isolation of so-called rosette positive cells or "rosetting" ensued. In this isolation technique, PBMCs are incubated with sheep erythrocytes. During this incubation an homologue of CD58, present on the surface of the sheep erythrocytes, binds CD2 which is expressed on the surface of cells expressing CD3 such as T cells and NK cells, but not on other mononuclear cells such as monocytes and B cells (Rosenberg et al., 1979). $10x10^6$/ml PBMCs were resuspended in RPMI1640 and 5 ml were dispensed per tube. 2.5 ml of FCS and 2.5 ml of sheep erythrocytes ($2x10^6$ cells/ml) were added to each tube, mixed and incubated for 10 min at 37°C while shaking in order to prevent deposition of the cells at the bottom of the tube. After incubation the cells were centrifuged for 10 minutes at 255 g and pellets were incubated for 45 min at 4°C. Afterwards, cells were gently resuspended, the content was combined for each two tubes and 10ml of Ficoll were layered at the bottom of each tube. Cells were centrifuged for 20 min, 400 g at room temperature, which allowed the formation of pellets constituted entirely of rosette-positive cells. Rosette-negative cells formed a thin layer above the Ficoll layer and were discarded by aspiration. Erythrocytes constituting, together with CD3 positive cells, the rosettes were lysed by vigorous resuspention of two pellets at a time with 10 ml of 155 mM NH_4Cl. Lysis was stopped by adding 40 ml of PBS. The remaining CD3 positive, rosette positive cells were washed with PBS and counted.

2.2.2.2 Isolation of CD4 naive and memory Th cells

Rosette positive cells were used for further isolation of either naive or memory Th cells, through a negative selection magnetic cell separation. Memory or Naive CD4+ T Cell

Isolation Kit II from Miltenyi Biotec were used. In this system, cells are bound by biotinylated antibodies directed against specific cell surface markers. The cells are then incubated with anti-biotin coated magnetic beads and run through a column fixed to a magnet. This allows flowthrough only of unlabeled cells, hence the negative selection. Both Memory or Naive CD4+ T Cell Isolation Kit II comprise antibodies against the following surface molecules: anti-CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, HLA-DR, glycophorin A, and TCR γ/δ. Additionally, the Naive CD4 + T Cell Isolation Kit II contains anti-CD45RO, thereby allowing exclusion of memory CD4 Th cells, while the Memory CD4+ T Cell Isolation Kit II comprises anti-CD45RA, allowing for retention of CD4 naïve Th cells inside the column. The rosette positive cells were re-suspended in MACS buffer at a concentration of 1 x 10^7 cells/ml, stained with 10 µl of antibody cocktail per 1 x 10^7 cells, and incubated for 10 minutes at 4°C. In the case of memory T cell isolation, incubation with the secondary antibody ensued, while for Naive T cells the cells were washed once with MACS buffer before adding 10 µl of anti-biotin MicroBeads per 1 x 10^7 cells. Cells were incubated for 15 minutes at 4°C and washed once with MACS buffer. Cells were then resuspended in 1 ml per 1 x 10^8 cells and 1 ml of cell suspention was loaded on a pre-wetted LS MACS column. After allowing flowthrough, the column was washed three times with 3 ml of ice-cold MACS buffer, counted, and 0.1 x10^6 cells were withdrawn for surface staining and purity check.

2.2.2.3 Differentiation of CD4 naive T cells

Freshly isolated CD4 naive Tconvs (3x10^6/well) or sorted Th1, Th2 or Th17 cells were cultivated for 5 days in 6 well plates coated with 1 µg/ml OKT3, in RPMI1640 medium supplemented penicillin G/streptomycin (50 units/ml), L-glutamine (2 mM) (all from Life technologies), human recombinant IL-2 (10 units/ml), 10% NHS, 1 µg/ml anti-CD28. This cocktail was supplemented with either anti-IL-4 for Th0-, IL-12 and anti-IL-4 for Th1, IL-4 and anti IFN-γ for Th2- and IL-1, IL-21, IL-23, anti-IL4 and anti-IFN-γ for Th17-inducing conditions. After 5 days of stimulation under these conditions, the cells were cultured for an additional 2 days in non- coated 6 well plates in the presence of IL-2. On day 7 the cells were harvested, washed, counted and either stimulated for 5 hours with PMA and ionomycin in the presence of monensin for intracellular staining, or re-stimulated for one hour with anti-CD3/anti-CD28, before the cytokine secretion assay was performed.

2.2.2.4 Cytokine secretion assay and sorting of T cell subsets

In order to sort pure populations of Th1, Th2 or Th17 cells, we stimulated either cultured

naive T cells that were differentiated under Th1, Th2 or Th17 inducing conditions, for 5 days plus two days in the presence of IL-2, or freshly isolated CD4 memory T cells over-night with anti-CD3 and anti-CD28. The next morning cells were harvested, washed and counted. Cells were then labeled with 167 µl/ml anti-IL-17A and anti-IFN-γ or anti-IL-4 and anti-IFN-γ antibodies, each conjugated to a leukocyte-specific CD45 antibody, incubated for 1 hour at 37°C on a MACS-mix rotor at a concentration of $1x10^6$ cells/ml to allow cytokine capture, and labeled with 167 µL/ml PE labeled anti-IL-17 or anti-IL4, and FITC labeled anti-IFN-γ (secretion assay - detection kits, Miltenyi Biotec), and sorted on a FACS Aria (BD Biosciences) or MoFlo (Beckman Coulter) (Figure 1). Purity of sorted populations was checked routinely by FACS and for some samples by qPCR.

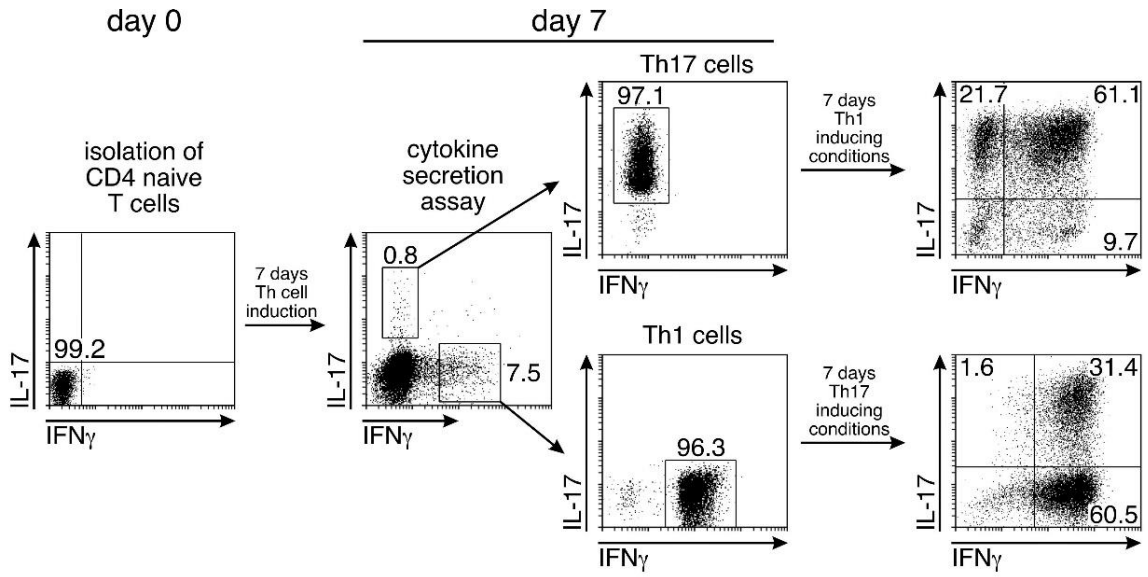


Figure 1. **Experimental setup.** Isolation of naive CD4 T cells, Th cell differentiation for 7 days, cytokine secretion assay followed by sorting of Th1 and Th2 effector cells, and transdifferentiation under different conditions for 7 days with subsequent cytokine analysis by flow cytometry.

2.2.3 Transdifferentiation

2.2.3.1 Transdifferentiation of CD4 Th cell subsets generated from naive T cells

Sorted *in vitro*-generated Th1, Th2 or Th17 cells were cultured for 5 days at a concentration of $0.25x10^6$ cells/ml in 96 well flat-bottom plates coated with 1 µg/ml OKT3, in RPMI1640 medium supplemented penicillin G/streptomycin (50 units/ml), L-glutamine (2 mM) (all from

Life technologies), human recombinant IL-2 (10 units/ml), 10% NHS, 1 µg/ml anti-CD28. This cocktail was supplemented with either anti-IL-4 for Th0-, IL-12 and anti-IL-4 for Th1, IL-4 and anti IFN-γ for Th2- and IL-1, IL-21, IL-23, anti-IL4 and anti-IFN-γ for Th17-inducing conditions. After 5 days of stimulation under these conditions, the cells were cultured for an additional 2 days in either 2.5 or 10ml polypropylene tubes with the addition of only IL-2. On day 7 the cells were harvested, washed, counted and stimulated for 5 hours with PMA and ionomycin in the presence of monensin in preparation for intracellular cytokine staining.

2.2.3.2 Transdifferentiation of CD4 memory T cells subsets

Freshly sorted *in vivo*-generated Th1, Th2 or Th17 ($0.01x10^6$/well) were cultured in OKT3 coated round bottom 96 well plates for 5 days in RPMI 1640 medium supplemented with L-glutamine, penicillin/streptomycin, 10% NHS and IL-2. Additionally cytokine and cytokine-neutralizing antibody cocktails were added for Th0, Th1, Th2, Th9 or Th17 inducing conditions, as described above. On day 5 the cells were harvested, washed, and counted. $0.01x10^6$ cells were taken from each sample for gene expression analysis. The remaining cells were plated in non-coated round bottom well plates at a maximum concentration of $1x10^6$ cells/ml in RPMI1640 supplemented with L-glutammine, penicillin G/streptomycin, 10% NHS, and IL-2 as previously described. After two days the cells were harvested, washed, counted and prepared for fixation.

2.2.4 Re-stimulation and Fixation

Cultured cells or over night-stimulated memory T cells were harvested, washed and counted. $1x10^6$ cells were plated in 1ml or RPMI1640 supplemented with L-glutammine, penicillin/streptomycin, 20 ng/ml of PMA, 1 nM ionomycin, and 2 µM monensin in a flat bottom 24 well plate for 5 hours at 37°C. After incubation, cells were washed once with PBS and stored in PBS at 4°C for further analysis. After re-stimulation, cells were washed once with PBS ad fixed with 3% PFA for 10 minutes at 37°C. After fixation the cells were washed twice with PBS and stored at 4°C for further analysis.

2.2.5 Flow cytometry

2.2.5.1 Flow cytometry of surface molecules

In order to verify the purity achieved during isolation, $0.1x10^6$ cells were washed with FACS buffer, resuspended in 50 µl FACS buffer and stained with 1 µl anti-CD3 and anti-CD4, anti-CD27, anti-CD45RA in case of isolated naive T cells and anti-CD27, anti-CD45RO in case of isolated memory T cells. Cells were incubated for 15 minutes at 4°C, washed with FACS buffer, resuspended in 200 µl FACS buffer and analysed on an FC500 Series flow cytometer by Beckman Coulter. A purity of ≥98 % was achieved for cells positive for both CD3 and CD4 (CD4 T cells). ~85% of the cells were positive for both CD27 and CD45RO (memory T cells).

2.2.5.2 Flow cytometry of intracellular cytokines

In order to determine the capacity of cultured cells to produce cytokines, intracellular staining was performed. After re-stimulation and fixation with PFA, cultured cells were washed twice with FACS-Saponin to allow permeabilization of the cells followed by 10 minutes of incubation with 4% mouse and rat sera at 4°C to block unspecific binding of antibodies. After an additional wash step with FACS-Saponin, cells were resuspended in 50 µl of FACS-Saponin and incubated for 30 minutes at 4°C with 0.1 µg (saturating amount) of anti-IL-1, -IL-4, -IL-9 and -IL-17. After incubation the cells were washed twice with FACS-Saponin and resuspended in 200 µl of FACS buffer. Analysis was performed on an FC500 flow cytometer (Beckman Coulter).

2.2.6 RNA isolation

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). $0.01x10^6$ cells per sample were lyzed by resuspension in 350 µl RLT buffer supplemented with 1% β-mercaptoethanol. The entire volume was then transferred to a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at full speed. The flow through was then transferred to a gDNA eliminator column and centrifuged at 8000 g for 30 seconds. 350 µl of 70% ethanol was added to the cell lysate and mixed by pipetting. The entire volume was then transferred to an RNeasy spin column placed inside the provided 2 ml collection tube. The column was centrifuged 30 seconds at 8000 g, flow through was discarded. Next the column was washed with 700 µl of buffer RW1 for 30 seconds at 8000 g. After discarding the flow through, the column was then washed twice with 500 µl RPE buffer for 30 seconds at 8000 g. The column

was placed in a new collection tube and centrifuged at 10000 g for 1 minute, in order to dry the membrane completely. Finally, 30 µl of RNase free water was pipetted on the membrane and incubated at room temperature for 1 minute, followed by elution of the column in a 1.5 ml tube by centrifugation at 10000 g for 1 minute. Due to the low cell number, it was not possible to measure RNA yield. The samples were stored at -80°C for further analysis.

2.2.7 Complementary DNA (cDNA) synthesis

Total RNA extracted from $0.01x10^6$ cells was reverse transcribed to cDNA using the Affinity Script QPCR cDNA Synthesis kit (Agilent Technologies) according to manufacturer's protocol. Briefly, 6 µl of RNA elute was pipetted in a PCR tube with a final volume of 20 µl containing 1x First strand mastermix, 12.75 nM Oligo(dT), 2.25 nM random primer, and 1 µl Affinity Script RT/RNase Block Enzyme mixture. This mix was then incubated at 25°C for 5 minutes, at 42°C for 5 minutes, at 55°C for 30 minutes, and at 95°C for 5 minutes. cDNA was frozen at -20°C for further analysis.

2.2.8 Real-time PCR

2.2.8.1 TaqMan Gene Expression assays

TaqMan Gene Expression assays were used to perform Real-time PCR with an ABI Prism 7000 Sequence Detection System (Life technologies). The assays include a pair of unlabeled PCR primers (For and Rev), a TaqMan probe which has a FAM or VIC dye label conjugated at the 5' end, and a minor groove binder (MGB) and a non-fluorescent quencher (NFQ) linked to the 3' end of the probe. During PCR, the probe anneals to the complementary sequence between For and Rev primers. However, Förster-type energy transfer prevents the reporter dye from fluorescing due to it's proximity to the NFQ. During the elongation phase of the PCR, the 5' nuclease activity of the polymerase excises the probe, thereby releasing the reporter dye which is free to fluoresce. This exponential increase of florescence can be detected by a quantitative PCR instrument. Each sample was analyzed in duplicates. 1 µl of cDNA was used for each PCR amplification, in a final volume of the mix was 20 µl. The mix consisted of 1x TaqMan Universal PCR mastermix and 1x TaqMan Gene Expression Assay mix. The samples were exposed to the following thermal cycling conditions: activation of the enzyme at 95°C for 10 minutes followed by 40 cycles of amplification each consisting of DNA denaturation at 95°C for 10 seconds followed by the annealing of primers and DNA extension at 60°C for 1 minute. The full list of genes analyzed by real-time PCR can be found

in the "materials" paragraph. Relative quantification was performed by calculating the difference in cross-threshold values (ΔCt) of the gene of interest and a housekeeping gene (*Cyclophilin*) according to the formula $2^{-\Delta Ct}$.

2.2.8.2 SYBR Green detection

In order to detect the histone modifications at the loci of interest, listed in the methods paragraph, real-time PCR was performed. The MacVector Version 12.0.2 software (Accelrys) was used to design the primers for ChIP analysis (For and Rev) for SYBR Green PCR. Amplified fragments were up to 120 base pair (bp) long. During PCR, the polymerase amplifies the target sequence and the SYBR Green dye is able to intercalate to each new copy of double stranded DNA, emitting fluorescence. Fluorescence intensity is thereby increased proportionally with the increase in the amount of PCR product. DNA samples were pre-diluted 1:5 to decrease pipetting error, then 5 µl of DNA template was pipetted in two (duplicates) or three (triplicates) wells for each sample. Each well was then filled with 15µl of the SYBR Green PCR reaction, which included 1x Power SYBR Green PCR master mix and 0.2 µM primer mix, for a final volume of 20 µl per well, in a 96 well plate. The amplification and detection of the fluorescence was conducted in an ABI Prism 7000 Sequence Detection System (Life technologies). The enzyme was activated at 95°C for 10 min prior to 40 cycles of amplifications. Each thermal cycle consisted of 10 seconds of denaturation at 95°C, 1 minute of annealing of the primers and DNA elongation at 60°C. After the last amplification cycle, a dissociation curve was built with the following thermal profile: 95°C for 15 seconds, 60°C for 30 seconds, 95°C for 15 seconds. The full list of genes analyzed by real-time PCR can be found in the "Materials" paragraph. Relative quantification was performed by calculating the difference in cross-threshold values (ΔCt) of the gene of interest and an input control (see the paragraph dedicated to the ChIP assays) according to the formula $2^{-\Delta Ct}$.

2.2.9 DNA amplification and gel electrophoresis

2.2.9.1 AmpliTaq DNA Polymerase PCR (Life technologies)

PCR amplification was performed in order to test cDNA integrity prior to performing real time PCR. 1 µl of cDNA was used in a final volume of 25 µl. The reaction contained 1x GeneAmp® PCR buffer I, 250 µM dNTPs, 0.5 µM cyclophilin Primer mix, and 0.5 units AmpliTaq® DNA Polymerase. The following thermal cycling parameters were applied: 95°C

5 min, 35 cycles at 95°C 30 sec, 60°C 30 sec, 72°C 1-2 min, and a final extension at 72°C for 7 min.

2.2.9.2 Gel electrophoresis to verify cDNA integrity

10 µl from each sample of PCR products was mixed with 2 µl of DNA 6X loading dye and loaded in a 1.8% agarose gel constituted by 50 ml TAE buffer, 0.9 g agarose and 10 µl SYBR Safe DNA gel stain. The electrophoresis was carried out in a horizontal chamber for 30 minutes at 100 V and 60 A. The integrity of the cDNA was analyzed through the use of a transilluminator (Herolab).

2.2.10 ChIP for histone modifications

The presence of specific histone modification patterns at specific genomic locations was assessed through ChIP assays carried out using the ChIP-IT High Sensitivity Kit (Active Motif). Up to 0,1 x 10^6 sorted Th1 and Th17 cells were re-suspended in 10 ml RPMI and cross-linked with 1% formaldehyde for 10 minutes. Fixation was stopped by 1.25M glycine for 5 minutes at room temperature. All centrifugation steps were performed at 723 g at 15°C for 5 min. After centrifugation, the cell pellet was washed with ice-cold PBS. Cells were then lysed in 250 µl cell lysis buffer, flash-frozen, re-suspended in nuclear lysis buffer and sonicated by a Bioruptor Next Generation (Diagenode**)** for two rounds of 20 cycles with 30 sec intervals, at 5°C. After sonication, the sheared chromatin was centrifuged for 10 min at 16,000 g at 4°C in order to let the cell debris sediment and supernatant was transferred to a new 1.5 ml tube. 50 µl were then transferred to new 1.5 ml Eppendorf tubes to de-crosslink and isolate the DNA fragments to use as input. Briefly, 250 mM NaCl, 40 µg of Proteinase K and 20 µg of RNase A were added to the 50 µl of sheared chromatin and incubated for 2 hours at 55°C followed by 30 min at 95°C to stop the reaction. The DNA was isolated using the GenElute PCR Clean-Up kit (Sigma-Aldrich) according to manufacturer's protocol. Briefly, 500 µl Column Preparation solution was added to each column. Then, 5 volumes of Binding solution were added to each chromatin sample and transferred to the column. Centrifugation steps were performed at 13,000 xg for 1 min. The DNA was bound to the column by centrifugation and washed once with 500 µl Wash solution. The column was dried and DNA was eluted in 32 µl Elution solution and used as Input control. In few preliminary experiments the entire elute, containing chromatin derived from between 0.05x10^6 and 1x10^6 were loaded with 6x Loading dye on a 2% agarose gel in order to check the fragment size

which, for the optimal results at the immunoprecipitation step, should range between 200 bp and 1000 bp.

ChIP reactions were performed with the remaining 200 µl of cell lysate, incubated over-night with 4 g of anti-H3K4me3, anti-H3ac, anti-H3K27me3, and rabbit IgG to assess background levels. After de-crosslinking and degradation of the chromatin structure (according to the ChIP-IT High Sensitivity Kit manufacturer's protocol), the eluted DNA was stored at -80°C until further analysis. The DNA samples were analyzed by quantitative PCR using the Power SYBR Green PCR Master Mix and 200nM primer mix for SYBR Green detection in the 7500 Fast Real-Time PCR System (Life Technologies). Primer pairs were used as follows: 5'-GGCAACCCGAAAGGTCACTTAG-3' and 5'-TTCTCCTGGCACTCAGAGGCTC-3' for the *T-bet* promoter region; 5'-TCTCCCCTATGCCTGTCACCTG-3' and 5'-TGATTTTGCCCAAGGACTCACAC-3' for the *RORC* promoter region. Data are presented as a log to the base 2 of the ratio of the immunoprecipitated amounts of DNA of H3K4me3 to H3K27me3 and H3ac to H3K27me3 pull-downs or as a ratio of immunoprecipitated DNA to that of input DNA. Binding specificity was verified by amplification of unspecific precipitated DNA with rabbit IgG.

2.2.11 Statistical analysis

Results were analyzed by Student's t-test (comparing two different groups). All calculations were carried out with Microsoft Excel. P-values less than 0.05 were evaluated as statistically significant and represented graphically as follows: * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, ns – not significant.

3 RESULTS

3.1 Transdifferentiation of *in vitro*-generated Th cell subsets

3.1.1.1 Differentiation of naïve CD4 T cells

CD4 naive T cells have the capacity, under TCR stimulation and exposure to the appropriate cytokine milieu, to differentiate into several different effector T cell subsets. In this study, Th1, Th2, and Th17 cells were generated from naive T cells as described in the methods. After Th1 induction, INFγ production was achieved on average by 43 ± 14.3% of the cells, with 38.3 ± 12.7% producing IFNγ but not IL-4, 39.6 ± 13.0% producing IFNγ but not IL-9, and 40.7 ± 14.0% producing IFNγ but not IL-17. 4.8 ± 2.9%, 3.4 ± 3.4%, and 2.4 ± 1.5% were instead IFNγ/IL-4, IFNγ/IL-9 and IFNγ/IL-17 double positive cells, respectively (Figure 2A). The frequency of cells producing IL-4 after Th2 induction was, on average, 12.5 ± 11.3% while those that were able to produce IL-4 but not IFNγ, IL-4 but not IL-9 and IL-4 but not IL-17 where 5.2 ± 5.7%, 11.4 ± 11.6%, and 10.9 ± 11.0%, respectively. The frequency of double producers, under these inducing conditions, was 7.7 ± 6.1% for IFNγ+IL-4+, 1.0 ± 0.8% for IL-4+IL-9+, and 1.3 ± 1.0% for IL-4+IL-17+ (Figure 2B). Finally, when cultured under Th17 inducing conditions, 19.8 ± 13.4% of the cells acquired the ability to produce IL-17. The frequency of "IL-17-only" producing cells were 9.3 ± 5.4% for IL-17+IFNγ-, 19.0 ± 13.2% for IL-17+IL-4-, and 18.9 ± 13.0% for IL-17+IL-9-. At the same time, 10.5 ± 10.0%, 0.8 ± 0.6%, and 1.1 ± 0.9% of IFNγ+IL-17+, IL-17+IL-4+, and IL-17+IL-9+ double producing cells, developed (Figure 2C). These results show that under all inducing conditions IFNγ+ cells developed while a moderate but significant percentage of naïve CD4 T cells could be induced to differentiate into Th2 and Th17 cells. Furthermore, under all conditions, cells capable of co-producing multiple cytokines arose, particularly cells positive for both IFNγ and IL-17, when under Th17 inducing conditions. Overall, all inducing conditions generated high frequencies of IFNγ producing cells. However, as expected the highest frequency of total IL-4- and IL-17-producing cells was achieved under Th2- and Th17-inducing conditions, respectively. Cells cultured under these inducing conditions were then used for cytokine secretion assay and sorting.

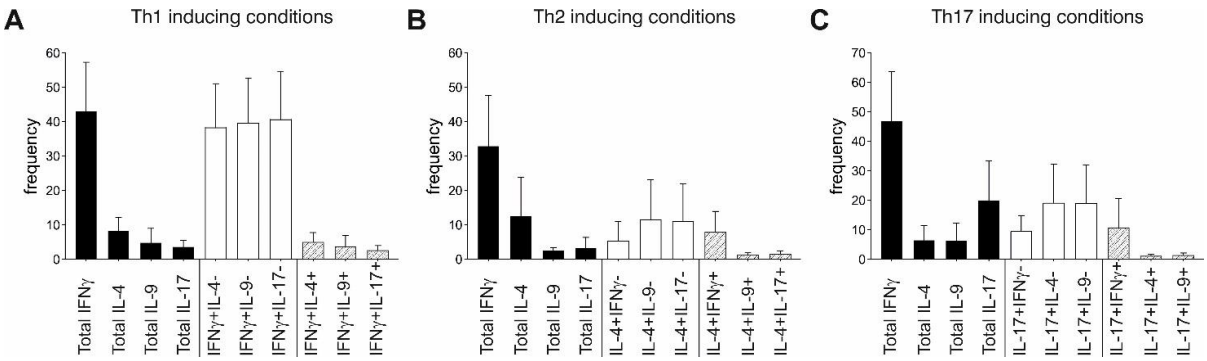


Figure 2. **Differentiation of naïve T cells.** Frequency of cytokine producing T cells after culture under (A) Th1- (anti-CD3, anti-CD28, IL-12, anti-IL-4), (B) Th2- (anti-CD3, anti-CD28, IL-4, anti-IFNγ), and (C) Th17-inducing conditions (anti-CD3, anti-CD28, IL-1, IL21, IL-23, anti-IL-4, anti-IFNγ), measured by intracellular cytokine staining and subsequent flow cytometric analysis.

3.1.1.2 Cytokine secretion assay and sorting of Th1, Th2 and Th17 cells

Naive CD4 T cells, after differentiation for 5 days and two days of resting, the cells were stimulated over night with anti-CD3 and anti-CD28 as described in the methods. The next day a cytokine secretion assay was performed, which allowed the sorting of highly pure, viable populations of Th1, Th2 or Th17 cells. Th1 cells were sorted by gating on IFNγ+ cells and excluding IL-17+ cells, Th2 cells were positive for IL-4 and negative for IFNγ while Th17 cells were selected as IL-17+IFNγ-. Due to technical restrictions, namely the lack of a cytokine secretion assay kit for IL-4 or IL-17 labeled in FITC, it was not possible to perform a CSA for these two cytokines at the same time. A CSA kit for IL-17 in APC, which would have allowed for the CSA of both IL-4 and IL-17, was tested but it resulted in low frequencies of IL-17 producing cells and consequently it yielded very low cell numbers upon sorting. Also, a surface staining for three cytokines was technically unfeasible. Nevertheless, Th2 cells, can still be defined as cells producing IL-4 and not IFNγ. Additionally, the frequency of cells producing both IL-4 and IL-17 is negligible in humans. For *in vitro*-generated Th1, Th2 and Th17 cells, the frequency of cells, following CSA, producing IFNγ, IL-4 or IL-17 were, on average, 6.4 ± 0.3% positive for IFNγ and negative for IL-17 after Th1 inducing conditions (Figure 3A) and 5.0 ± 3.0% after Th17 inducing conditions (Figure 3C). IL-17+IFNγ- cell frequency was instead 0.3 ± 0.3% in cells cultured under Th1 inducing conditions (Figure 3A) and 1.2 ± 0.6% under Th17 inducing conditions (Figure 3C). On the other hand, the frequency of Th2 cells under Th2-inducing conditions was 0.9 ± 1.1% (Figure

3B). In order to limit the amount of impurities in the sorted samples, very restrictive gating was applied before sorting. This, together with the events discarded by the sorter due to the so called "electronic aborts" and to the cells dying due to the stress induced by the sorting process, led to a considerably lower yield of sorted cells than would otherwise be expected based on the pre-sorting frequencies. The yield of the sorted, *in vitro*-generated Th1, Th2 and Th17 cells was on average 0.137 x 10^6 of Th1 cells and 0.015 x 10^6 for Th17 cells under Th1 inducing conditions, 0.051 x 10^6 of Th2 cells under Th2 inducing conditions, and 0.388 x 10^6 of Th1 cells and 0.151 x 10^6 for Th17 cells under Th17 inducing conditions. The *in vivo*-generated cells underwent a more physiological overnight stimulation with anti-CD3 and anti-CD28. Consequentially, these cells showed lower frequencies of cytokine producing cells upon CSA and even lower yields of sorted Th1, Th2 and Th17 cells. IFNγ+IL-17- cells were on average 2.9 ± 1.6% (Figure 4A). The frequency of IFNγ-IL-17+ (Th17) was higher in memory cells from RA compared to HCs (0.5 ± 0.3% vs. 0.1 ± 0.1%, p=0.0001) (Figure 4A). The frequency of IFNγ-IL-4+ (Th2) was instead 0.1 ± 0.1%, with no differences between RA and HCs (Figure 4B). For the same reasons mentioned for the *in vitro*-generated T cell subtypes, also in this case the yield of sorted cells was low, with on average 0.058 x 10^6 Th1, 0.005 x 10^6 Th2, and 0.007 x 10^6 Th17 cells sorted. Because of these low numbers of sorted cells, not all of the subsequent experiments could be performed with the same samples, starting from the same cells. Additionally, for some experiments (i.e. ChIP assays) cells deriving from different donors had to be pooled together.

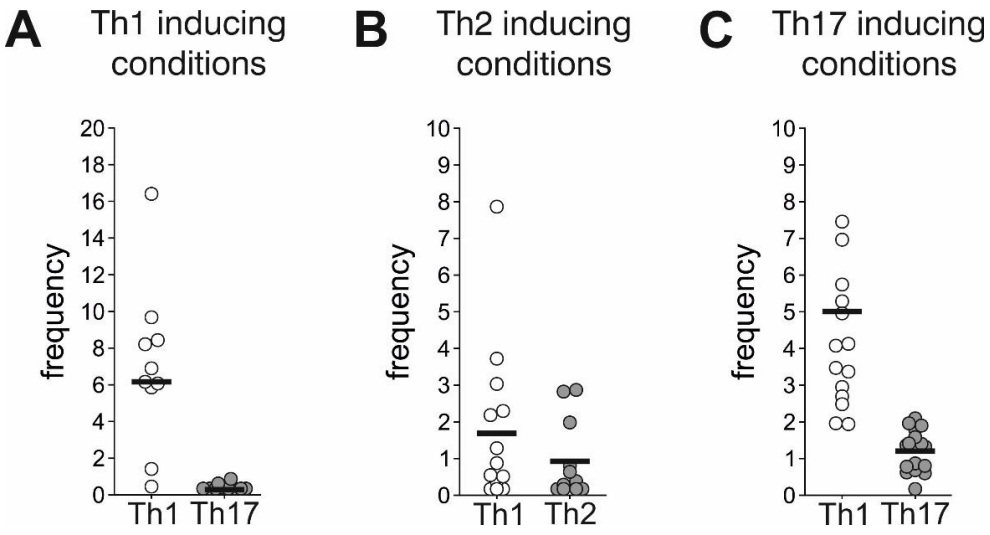


Figure 3. **Th cell frequencies of *in vitro*-generated Th cells after cytokine secretion assay and before sorting.** Frequency of cytokine producing memory CD4 T cells (according to CSA) after culture under (A) Th1, (B) Th2-, and (C) Th17-inducing conditions, assessed by flow cytometry after surface staining.

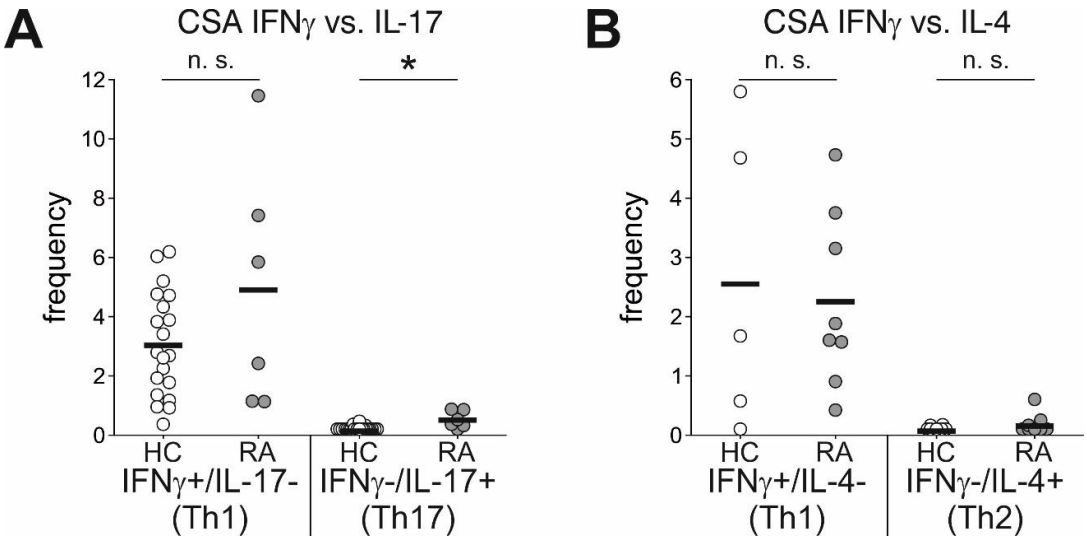


Figure 4. **Th cell frequencies of *in vivo*-generated Th cells after cytokine secretion assay and before sorting.** Frequency of cytokine producing T cells (according to CSA) after isolation of CD4 memory T cells and overnight stimulation. (A) IFNγ+/IL-17- (Th1) cells and IFNγ-/IL-17+ (Th17) cells in HC and RA. (B) IFNγ+/IL-4- (Th1) cells and IFNγ-/IL-4+ (Th2) cells in HC and RA, assessed by flow cytometry after surface staining.

3.1.1.3 mRNA expression analysis for purity control

Due to the low yield of sorted cells, it was not possible to check the purity of the cells by performing re-stimulation and intracellular cytokine staining. In order to check purity, $0.003x10^6$ freshly sorted cells were run in our FC500 Series Beckman Coulter flow cytometer. The average purity of the sorted *in vitro*-differentiated Th cells was 95.9 ± 3.1% for IFNγ+IL-17-, 96.6 ± 0,5% for IL-4+IFNγ- and 93.9 ± 1,8% for IFNγ-IL-17+ (Figure 5A). For *in vivo*-differentiated cells the average purity was 95.9 ± 3,6% for IFNγ+IL-17-, 87.2 ± 4,8% for IFNγ-IL-17+ (Figure 5B). In this case it was not possible to evaluate the purity of Th2 cells with this system as the average number of sorted cells was too low.

Additionally, purity was evaluated through the analysis of the levels of expression of specific cytokine encoding genes associated with each Th subtype, namely IFNγ for Th1, and IL-17 for Th17 cells. When comparing IFNγ and IL-17 expression in sorted *in vivo*-generated Th1 and Th17 cells, a clear difference was observed with virtually no IL-17 production by sorted Th1 cells, which in turn produced high levels of IFNγ, and vice versa no IFNγ production by Th17 cells, which instead produced high levels of IL-17 (Figure 5C).

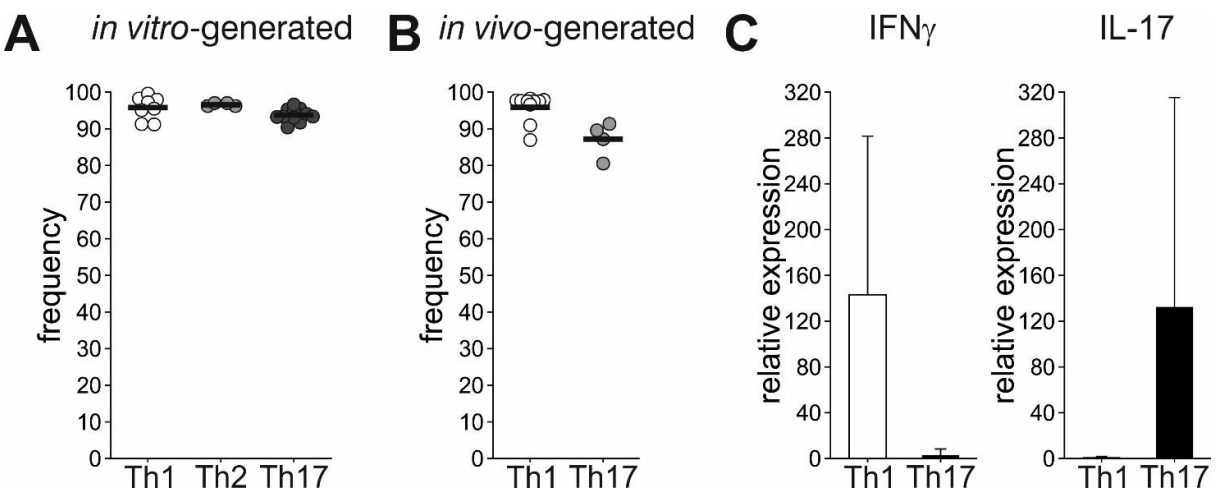


Figure 5. **Purity control of *in vivo*- and *in vitro*-generated Th cells.** Purity of cytokine producing T cells (according to CSA) after (A) culture under Th1-, Th2-, and Th17-inducing conditions and sorting. (B) Purity of sorted *in vivo*-generated Th1 and Th17 cells, assessed by flow cytometry after sorting. (C) Cytokine mRNA expression of sorted Th1 and Th17 cells, as measured by qPCR.

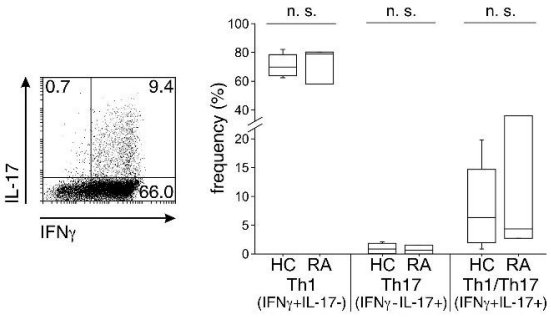
3.1.1.4 Transdifferentiation of in vitro-generated Th subsets

Plasticity has been reported to be a common phenomenon in mouse and human, both *in vitro* and *in vivo*. However, its role in the pathogenesis of rheumatoid arthritis is not yet well understood. In this study, a comparison of the plastic capacity of Th subtypes derived from healthy individuals and RA patients was made. We hypothesized that a supposed difference in plasticity between Th1, Th2 or Th17 derived from HC or RA would be due to an intrinsic, possibly genetic, characteristic of the RA Th cell. Therefore, for our first set of experiments we focused on testing the plastic capacity of in vitro-generated Th1, Th2 and Th17 cells. The experimental setup consisted of the isolation of naive CD4 T cells from peripheral blood of healthy donors and RA patients, the differentiation under Th1, Th2 and Th17 inducing conditions for 5 days with two additional days of resting with IL-2. On day 7 the cells were TCR stimulated overnight and, on the next day, part of the cells were withdrawn for re-stimulation and intracellular cytokine staining, while the rest of the cells underwent a cytokine secretion assay which allowed sorting of highly pure, viable Th1, Th2 and Th17 cells. In order to test their plastic capacity 0.05 x 10^6 sorted Th1, Th2 and Th17 cells, derived from PB of healthy donors and RA patients, were cultured under Th0- (neutral), Th1-, Th2-, Th9- or Th17-inducing conditions for 5 days with 2 additional days of resting. On day 14, cells were re-stimulated for 5 hours with PMA and ionomycin in the presence of monensin, fixed and

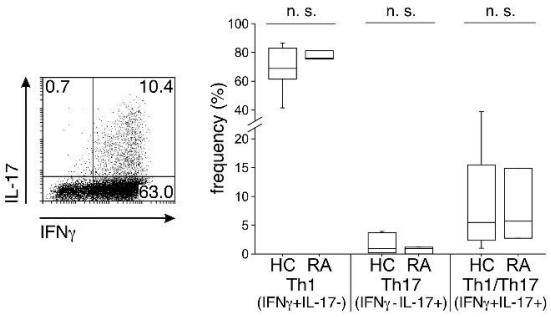
stained intracellularly with saturating amounts of directly labeled antibodies against IFNγ, IL-4, IL-9 and IL-17.

In vitro-generated Th1 cells largely retained their phenotype, defined by the expression of IFNγ but not IL-17 (IFNγ+IL-17-), upon transdifferentiation. Less than 5 % of the cells were able to convert to the Th17 phenotype (IFNγ-IL17+) under all inducing conditions (Fig. 6 A – E, left box plots). A larger portion of Th1 cells (≈ 5-10%) converted to a Th1/Th17 phenotype, characterized by the simultaneous expression of both IFNγ and IL-17 (IFNγ+IL-17+), under Th0-, Th1-, Th2- and Th9-inducing conditions (Figure 6A and B, right box plots, C and D, bottom panels, right box plots). This conversion was even more pronounced when the cells were cultured under Th17-inducing conditions (≈ 30%, Figure 6E, right column). Under Th2- and Th9-inducing conditions around 15% and 10% of *in vitro*-generated Th1 cells acquired the ability to produce IL-4 and IL-9, respectively (Fig. 6D, E, upper panels, left and middle columns). No significant differences in the plastic capability of *in vitro*-generated Th1 cells to convert into IL-4-, IL-9- and IL-17-producing phenotypes were observed, between HC and RA patients. *In vitro*-generated Th2 cells, cultured under Th17-inducing conditions, were not as able as the Th1 cells to retain their phenotype and resist transdifferentiation. In fact, the majority of Th2 cells acquired the expression of IFNγ, in both HC and RA patients (total IFNγ+: ≈ 64%, not shown in figure). Th2 cells, cultured under Th17 conditions, resulted in lower percentage of cells bearing the Th17 phenotype, compared to Th1 cells under the same inducing conditions (≈ 1.5 %, Figure 6F, lower panel, left). Moreover, in contrast to Th1, cells only a small population of Th2 cells acquired the Th1/Th17 phenotype (≈ 4 %, Figure 6F, lower panel, right), and cells with a Th2/Th17 phenotype expressing IL-17 in addition to IL-4 were rarely generated (≈ 2,5%, Figure 6F, upper panel, left). Also in the case of in vitro-generated Th2 cells, no significant differences in plasticity towards other phenotypes were evident between HC and RA patients. Together, while *in vitro*-generated Th1 cells are able to transdifferentiate towards a Th1/Th17 phenotype and *in vitro*-generated Th2 cells are capable of acquiring IFNγ-producing capabilities, both demonstrate low plasticity towards an IL-17-only producing Th17 phenotype. However, for both cell types, no significant differences in their plastic capability were observed between HC and RA. In conclusion, *in vitro*-generated Th1 and Th2 cells demonstrated similar plasticity towards the Th17 phenotype in healthy individuals and RA patients.

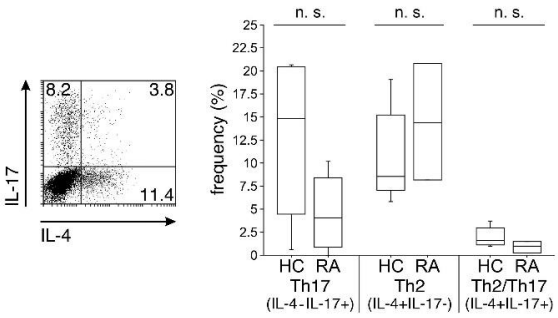
A Th1 under Th0-inducing conditions

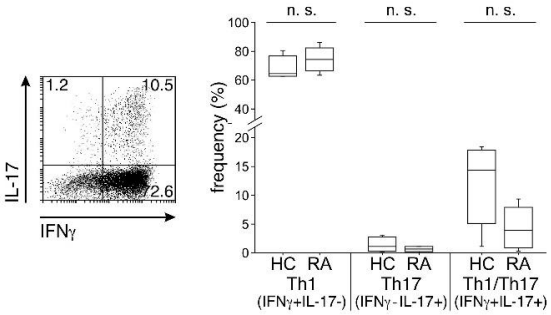


B Th1 under Th1-inducing conditions

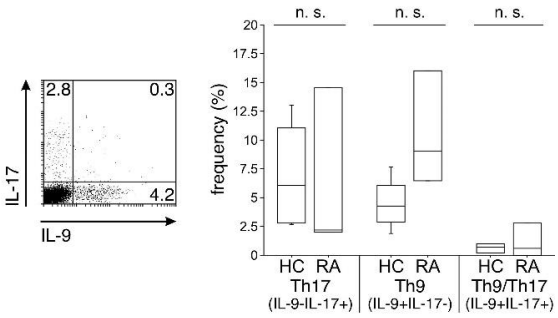


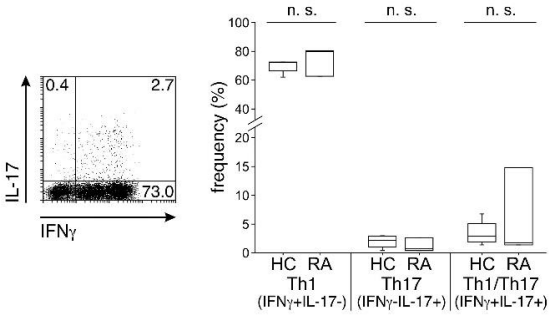
C Th1 under Th2-inducing conditions



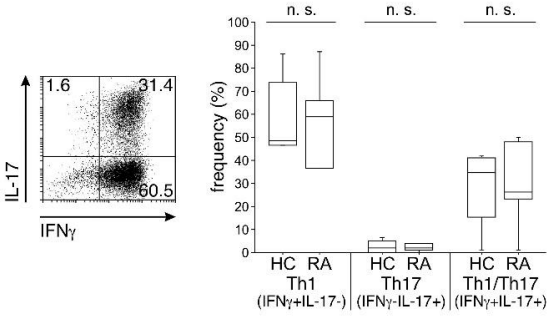


D Th1 under Th9-inducing conditions

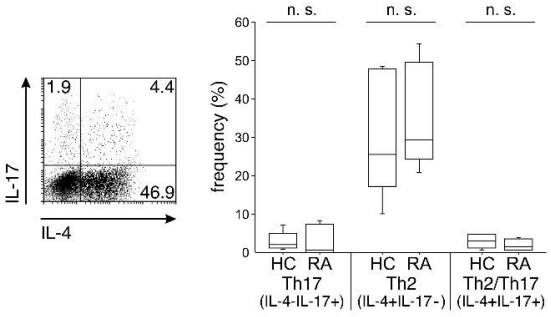




E Th1 under Th17-inducing conditions



F Th2 under Th17-inducing conditions



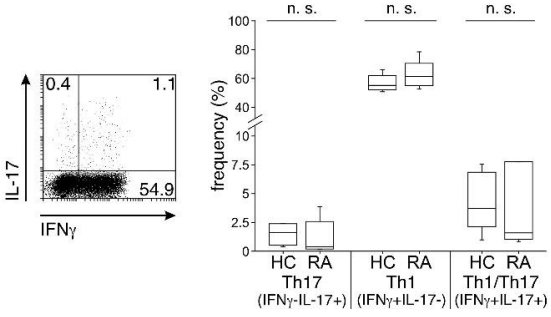


Figure 6. **Similar plasticity of *in vitro*-generated Th1 and Th2 cells towards Th17 phenotypes in healthy individuals and RA.** (A) Experimental setup with isolation of naive CD4 T cells, Th cell differentiation for 7 days, cytokine secretion assay followed by sorting of Th1 and Th2 effector cells, and transdifferentiation under different conditions for 7 days with subsequent cytokine analysis by intracellular staining and flow cytometry. (A-F) Cytokine expression after transdifferentiation demonstrated as representative stainings (left) and summary for healthy controls (HC, n=4-5 and patients with rheumatoid arthritis (RA, n=3-7) as box-and-whisker plots (right) for the transdifferentiation of Th1 cells under Th0- (A), Th1- (B), Th2- (C), Th9- (D), and Th17-inducing conditions (E). Th2 cells cultured under Th17-inducing conditions (F, upper panel) and expression of IL-4 against IL-17 against IL-4 (F, lower panel). n.s. - not significant.

3.1.1.5 Similar plasticity of in vitro-generated Th17 cells in healthy individuals and RA

In order to investigate the plasticity of in *in vitro*-generated Th17 cells we tested their potential to convert to a Th1, Th2 phenotype or to retain their Th17 phenotype in HC and RA patients. Therefore, *in vitro*-generated Th17 cells were transdifferentiated under Th1-, Th2- and Th17- inducing conditions. Under Th1-inducing conditions, very few Th17 cells were able to convert to an IFNγ-only producing phenotype, demonstrating a rather low plastic ability by these cells to perform a total conversion to Th1 cells, in both HC and RA (≈ 17%, Fig. 7A, left column). On the other hand, only a minority of *in vitro*-generated Th17 were able to maintain their full Th17 phenotype, while a large portion shifted towards a Th1/Th17 phenotype in both HC and RA patients (≈ 60 %, Fig. 7A, right). However, under Th2-inducing conditions, these Th17 cells demonstrated a very low degree of plasticity towards IL-4 and IL-4/IL-17 producing cells, with most of them remaining positive exclusively for IL-17 when stained against IL-4 (Figure 7B upper panel, left, middle). Finally, *in vitro*-generated Th17 cells were cultured under Th17-inducing conditions. As expected, under Th17- compared to Th1-inducing conditions Th17 cells from HC and RA demonstrate less plasticity towards the Th1 phenotype (Th17 ≈ 5% vs. Th1 ≈ 17%) (Figure 7C left, middle). Taken together these data indicate that *in vitro*-generated Th17 cells are prone to transdifferentiate to the Th1/Th17 phenotype, demonstrating similar plasticity between HC and RA patients.

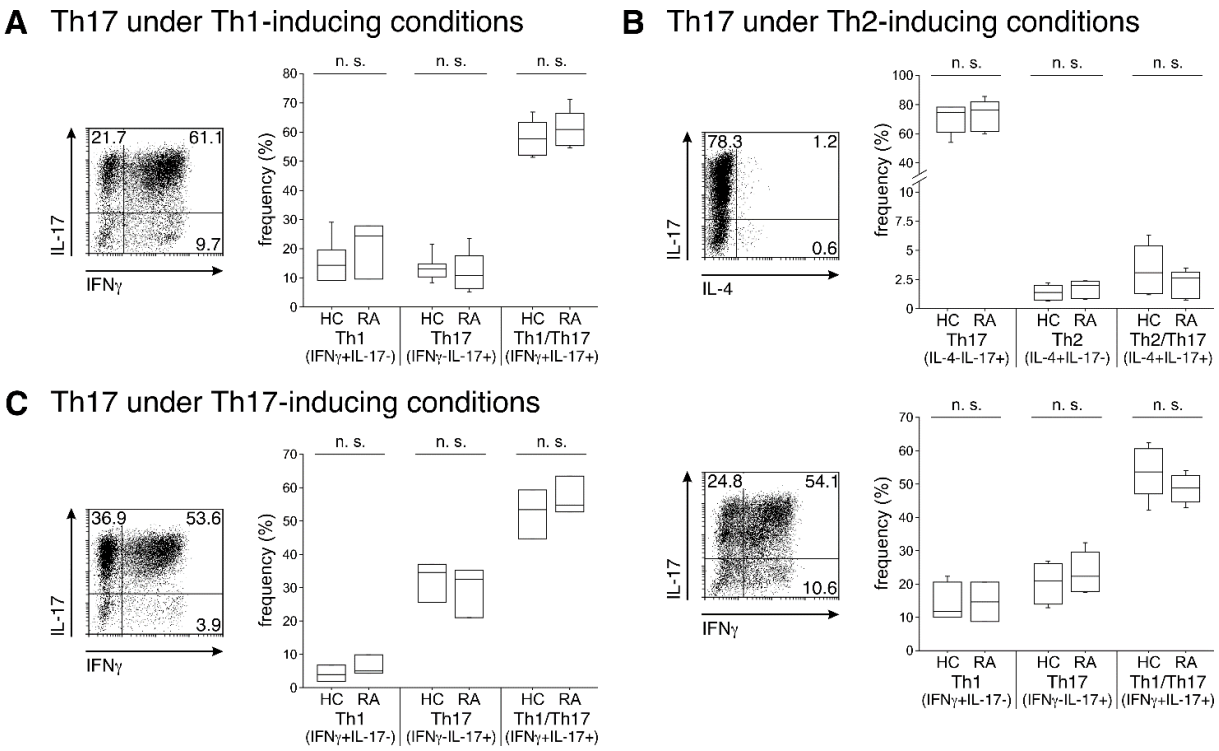


Figure 7. Similar plasticity of *in vitro*-generated Th17 cells in healthy indivduals and RA. Th17 effector cells generated *in vitro* from naive CD4 T cells were sorted and cultured under Th1-, Th2- and Th17-inducing conditions. Cytokine expression of IL-17 and IFNγ after transdifferentiation is demonstrated as representative intracellular stainings (left) and flow cytometry summary (right) for healthy controls (HC, n=3-7) and patients with rheumatoid arthritis (RA, n=3-5) shown as box-and-whisker plots for the transdifferentiation of Th17 cells under Th1-inducing conditions (A), under Th2-inducing conditions (B, upper panel) as well as expression of IL-17 against IL-4 (B, lower panel), and under Th17-inducing conditions (C). n.s. - not significant.

3.1.1.6 Enhanced transdifferentiation of in vivo-generated RA Th1 and Th2 cells towards a Th17 phenotype

Having observed similar plasticity by Th cells generated *in vitro* from HC and RA patients, we hypothesized that an altered plasticity of *in vivo*-generated Th cells might favor the predominance of Th17 cells observed in RA. We first tested whether *in vivo*-derived RA Th1 and Th2 cells might have an increased plasticity towards Th17 cells. To this goal, effector T cells were isolated *ex vivo* and sorted using cytokine secretion assay after a short TCR activation. This allowed for the sorting of highly pure, viable Th1 and Th2 cells. Similar to *in vitro*-generated Th1 cells, *in vivo*-derived Th1 cells from both HC and RA largely retained the Th1 phenotype under all conditions, namely neutral (Th0), Th1-, Th2-, Th9-, and Th17 inducing conditions (8A-E). Interestingly, not only under Th17- but also under all other inducing conditions, *in vivo*-derived Th1 cells from RA patients demonstrated a significantly

higher plasticity towards the Th17 phenotype, compared to those from HC (Figure 8A-E, RA vs HC; Th0: 1.0 ± 0.5 vs. 0.4 ± 0.3%, p=0.09; Th1: 0.8 ± 0.4 vs. 0.3 ± 0.3%, p=0.002; Th2: 1.3 ± 1.0 vs. 0.4 ± 0.2%, p=0.001; Th9: 2.7 ± 1.2 vs. 0.5 ± 0.3%, p=0.002; Th17: 2.3 ± 1.0 vs. 0.9 ± 0.9%, p=0.0002). Similarly to the previous result, also the *in vitro*-generated Th1 cells that didn't shift towards a full Th17- phenotype, but rather converted to the mixed Th1/Th17 phenotype, demonstrated a higher tendency to do so in RA compared to HC, independent of the inducing conditions (Figure 8A-E, RA vs HC; Th0: 11.7 ± 4.4% vs. 4.0 ± 3.9%, p=0.04; Th1: 7.9 ± 5.1 vs. 2.5 ± 2.2%, p=0.006; Th2: 9.4 ± 5.7 vs. 2.7 ± 2.3%, p=0.0003; Th9: 8.0 ± 3.6 vs. 2.0 ± 2.4%, p=0.009; Th17: 17.5 ± 11.2 vs. 7.9 ± 7.9%, p=0.008;). Similarly to *in vitro*-generated Th1 cells, when evaluating the plasticity of *in vivo*-derived Th1 cells, maintenance of the Th1 phenotype was observed under Th2-inducing conditions, with ≈ 62 % of the cells expressing IFNγ but not IL-4 (data not shown). However, the frequency of *in vitro*-generated Th1 cells cultured under Th2-inducing conditions which acquired concurrent IL-4 and, despite Th2 conditions, IL-17 expression, was significantly higher in RA compared to HC (Figure 8C, upper panel, right; 1.7 ± 1.6 vs. 0.7 ± 0.4%, p=0.04). Also, the Th1 cells from RA patients converted more to an IL-17 positive, IL-4 negative phenotype in RA compared to HC, under Th2 inducing conditions (Figure 8C, upper panel, left; 9.1 ± 6.1 vs. 2.4 ± 2.2%, p=0.0005). The same was true for cells expressing IL-17 but not IL-9, after Th9 inducing conditions (Figure 8D, upper panel, left; 9.8 ± 4.9 vs. 2.0 ± 2.4%, p=0.008). IL-9 positive, IL-17 negative Th9 cells were instead reduced in RA compared to HC (Figure 8D, upper panel, middle; 40.1 ± 20.2 vs. 17 ± 12.3, p=0.045). Finally, IL9/IL-17 double-positive cells, were found at an extremely low frequency and with no differences between HC and RA (Figure 8D, upper panel, right). Sorted *in vivo*-generated Th2 cells largely maintained their IL-4-producing phenotype, when cultured under Th17 inducing conditions, in both HC and RA (42.6 ± 19.6% and 46.1 ± 15.5%; Fig. 8F, upper panel, middle). However, the frequency of both cells fully converted to a Th17 phenotype and those acquiring IL-17 production in addition to IL-4 production (Th2/Th17), were significantly higher in RA compared to HC (20.7 ± 19.8% vs. 2.5 ± 1.7%, p=0.05, and 5.5 ± 1.6% vs. 2.9 ± 1.8%, p=0.04, respectively; Fig. 8F, upper panel, left and right). Therefore, RA *in vivo*-generated Th2 cells, show a tendency towards IL-17 expression when cultured under Th17 inducing conditions. However, despite the Th17 inducing conditions, ≈ 30% of the Th2 cells started to express IFNγ (data not shown). Consistent with the shift of Th2 cells from RA patients toward Th17 phenotypes, the frequency of cells that instead converted to Th1 cells (IFNγ+IL-17-) was decreased in RA (28.8 ± 10.6% vs. 14.3 ± 4.8%, p=0.02; Figure 8F, bottom panel, left), while the frequency of cells expressing IL-17 but not IFNγ, and cells expressing both cytokines, tended to be higher

in RA compared to HC (IFNγ-IL-17+: 10.0 ± 7.4% vs. 3.9 ± 1.8%, p=0.05; IFNγ+IL-17+: 15.0 ± 17.1% vs. 2.2 ± 1.3%, p=0.1; Figure 8F, bottom panel, center and right). In conclusion, these results indicate that *in vivo*-derived Th1 and Th2 cells from RA patients show a tendency to plastically shift towards IL-17 production, independent of the culture conditions.

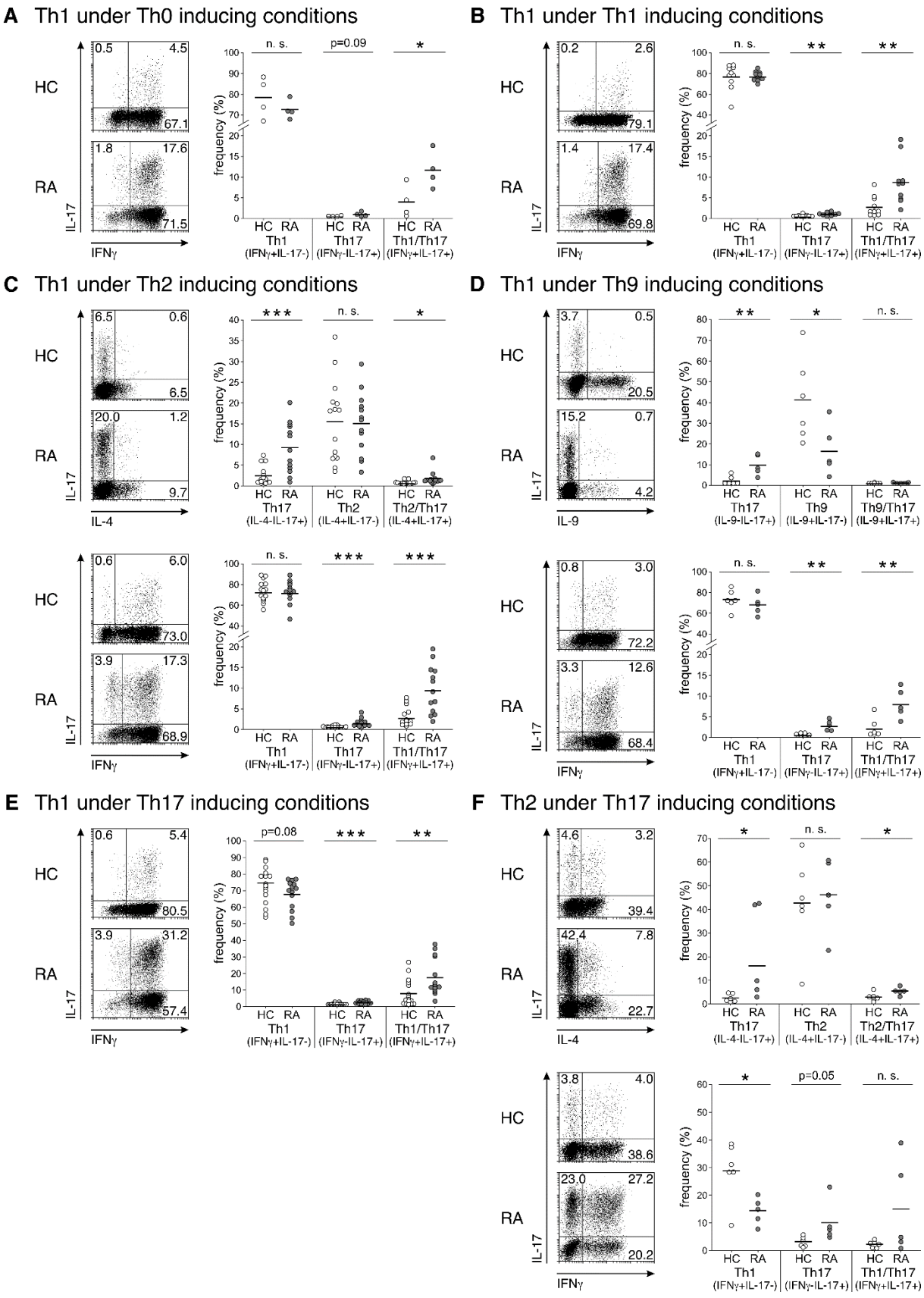


Figure 8. Enhanced transdifferentiation of *in vivo*-generated RA Th1 and Th2 cells towards a Th17 phenotype. Th1 and Th2 effector cells sorted from memory CD4 T cells were transdifferentiated under different inducing conditions. Intracellular cytokine staining and flow cytometry were performed. Cytokine expression of

IL-17 and IFNγ after transdifferentiation is demonstrated as representative stainings (left) and summary (right) for healthy controls (HC, n=4-18) and patients with rheumatoid arthritis (RA, n=4-14) shown as dots representing individual experiments for Th1 cells cultured under Th0- (A), Th1- (B), Th2- (C), Th9- (D), and Th17-inducing conditions (E). Th2 cells cultured under Th17-inducing conditions with expression of IL-4 against IL-17 (F, upper panel) and IFNγ against IL-17 (F, lower panel). n.s. - not significant. Mean is shown as a horizontal line. n.s. - not significant, * - $p<0.05$, **- $p<0.01$, ***- $p<0.001$

3.1.1.7 In vivo-generated Th17 cells from RA patients are resistant to transdifferentiation

Next, we investigated whether the increased frequency of Th17 cells observed in the peripheral blood of RA patients, might also be due to a resistant phenotype of these cells to plastically shift towards different T cell subtypes in RA. In order to test this hypothesis, *in vivo*-differentiated Th17 cells were isolated and cultured under either Th1 or Th2 inducing conditions. In this case, it was unfortunately not possible to apply the other inducing conditions, namely neutral (Th0), Th9 and Th17, as the number of sorted Th17 cells was too scarce, due to the low frequency of this T cell subtype in the context of cytokine secretion assay.

In vivo-generated Th17 cells cultured under Th1 inducing conditions behaved very differently between HC and RA. In fact, while Th17 cells from HC readily shifted their pattern of cytokine production towards a total conversion to Th1 cells, cells derived from RA largely maintained their IL-17-only producing Th17 phenotype (IFNγ-IL-17+: 20.3 ± 6.8% vs. 28.9 ± 6.4%, p=0.02; Figure 9A, center). On the other hand, the cells that fully converted to Th1 cells were significantly less in RA compared to HC (18.4 ± 5.1% HC vs. 10.0 ± 5.8% RA, p=0.008; Figure 9A, left). However, Th17 cells derived from both HC and RA acquired IFNγ production in addition to IL-17 production to a similar extent (48.6 ± 8.2% vs. 51.1 ± 8.2% p=0.53; Figure 9A, right).

Although no significant differences were observed between HC and RA in the IL-17 production capability of Th17 cells cultured under Th2 inducing conditions, significantly less Th17 cells from RA were able to convert to an IL-4 positive, IL-17 negative phenotype compared to HC (2.6 ± 1.2% HC vs. 0.9 ± 0.5% RA, p=0.017; Figure 9B, upper panel, center).

Together, these data indicate a resistance of *in vitro*-generated Th17 cells derived from RA patients to convert into Th1 or Th2 cells, compared to HC.

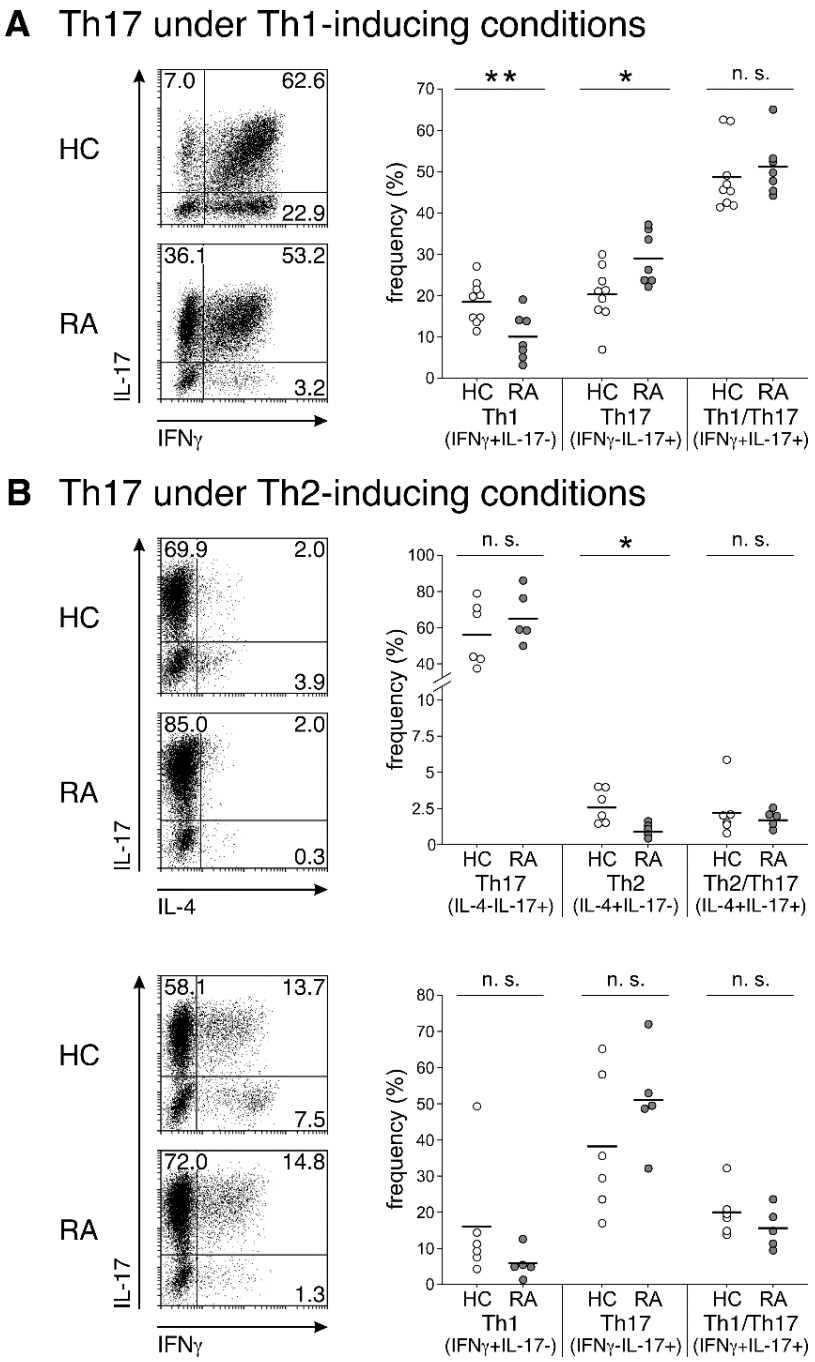


Figure 9. **Partial resistance of *in vivo*-generated Th17 cells from RA patients to transdifferentiate**. Th17 effector cells differentiated from memory CD4 T cells were sorted and transdifferentiated under different inducing conditions. Intracellular cytokine staining and flow cytometry were performed. Cytokine expression of IL-17 and IFNγ after transdifferentiation is demonstrated as representative stainings (left) and summary (right) for healthy controls (HC, n=6-9) and patients with rheumatoid arthritis (RA, n=5-7) shown as dots representing individual experiments for transdifferentiation of Th17 cells under Th1-inducing conditions (A), and under Th2-inducing conditions (upper) as well as expression of IL-17 against IL-4 (B, lower panel). n.s. - not significant. Mean is shown as a horizontal line. n.s. - not significant, * - $p<0.05$, **- $p<0.01$, ***- $p<0.001$

3.2 Gene expression analysis

3.2.1.1 Enhanced RORC gene expression in in vivo-derived Th cells upon transdifferentiation

Having observed differences in the pattern of cytokine expression at the protein level, between HC and RA, of Th cell subsets upon transdifferentiation, we sought to verify whether these differences would manifest also at the mRNA level already in the freshly isolated in vivo-generated cells, or only after further culture. For this reason we performed TaqMan gene expression analysis on INFγ, IL-4, and IL-17 in sorted Th1 and Th17 cells before and after transdifferentiation. It was not possible to include gene expression analysis of sorted Th2 cells as the number of sorted cells was too low.

Sorted Th1 cells expressed, as expected, high levels of IFNγ in both HC and RA (Figure 10A, left panel, left). IL-4 was instead expressed at very low levels in Th1 cells, with no differences between HC and RA (Figure 10A, left panel, center). A slight tendency for higher IL-17 gene expression in RA compared to HC was observed, although this was not statistically significant (Figure 10A, left panel, right). A similar gene expression status was found in sorted Th17 cells, with no significant statistical differences between HC and RA (Figure 10A, right panel). Of note, IFNγ and IL-4 expression was very low, as expected in sorted Th17 cells, while IL-17 mRNA expression was high and with a tendency for even a slightly higher expression per cell in Th17 cells from RA, compared to HC (Figure 10A, right panel). Overall, the lack of differences in cytokine gene expression between HC and RA is not of surprise, given that the cells were gated similarly for sorting in the case of both HC and RA patient-derived, *in vivo*-generated T cells.

In an attempt to find possible underlying mechanisms for the differences in plasticity previously described between HC and RA, we extended this analysis not only to the cytokine genes, but also to genes encoding for the master transcription factors of Th1, Th2 and Th17 cells, TBX21, GATA3 and RORC, respectively. While it was expected that TBX21 would be well expressed in Th1 cells, RORC expression was surprisingly high (Figure 10B, left panel, left and right). However, both transcription factors did not differ in their level of expression between HC and RA. Additionally, there was also no difference in the expression levels of GATA3 between HC and RA, in Th1 cells (Figure 10B, left panel, center). Finally, a very similar gene expression status was found for these genes in isolated, *in vivo*-generated Th17 cells. In this case TBX21 expression was, surprisingly, only slightly lower in Th17 cells compared to Th1 cells. There were, however, no differences between Th17 cells derived from HC and RA (Figure 10B, right panel, left). RORC was, as expected, more expressed in Th17

cells compared to Th1 cells, but again there were no differences in the expression level between cells derived from HC, compared to RA (Figure 10B, right panel, right). GATA3 was expressed at surprisingly high levels in both HC and RA, especially considering the extremely low amounts of IL-4 expressed by these cells (Figure 10B, right panel, center). Taken together these results show that *in vivo*-generated Th1 and Th17 cells have the potential to transdifferentiate towards other lineages, as demonstrated by their co-expression of the master transcription factors (TBX21, GATA3 and RORC) of the opposing Th cell lineage. However, no significant differences were detected between cells originating from HC and RA patients.

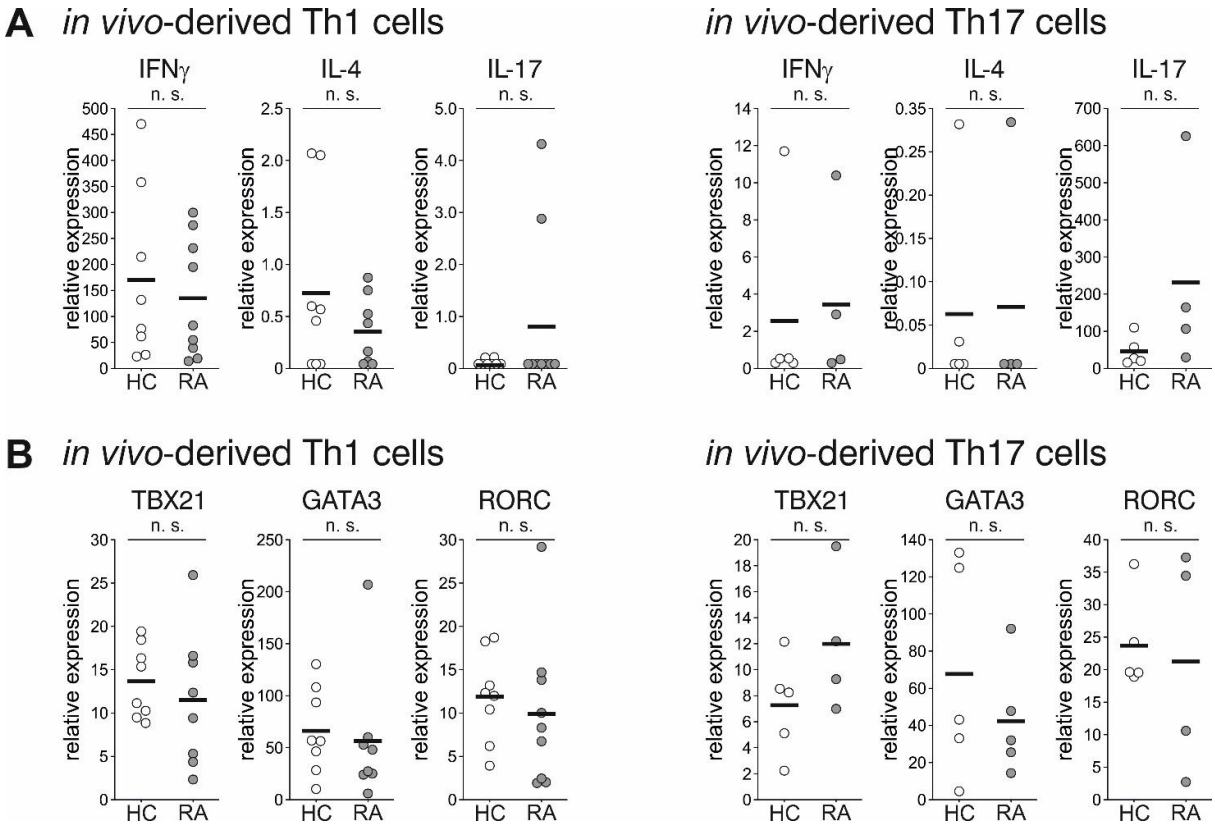


Figure 10. Expression of cytokine and transcription factors in freshly sorted, *in vivo*-derived Th1 and Th17 cells. Relative gene expression assessed by qPCR in *in-vivo* derived Th1 and Th17 cells for (A) IL-17, IFNγ, and IL-4 cytokine encoding genes, and (B) RORC, TBX21 and GATA3 transcription factors in HC (HC Th1: n = 8, Th17: n = 5) and patients with rheumatoid arthritis (RA, Th1, n = 8-9, Th17, n = 4-5).

Since we did not detect any differences between HC and RA in the gene expression levels, particularly of the master transcription factors TBX21 and RORC in freshly isolated *in vivo*-

generated Th1 and Th17 cells, we hypothesized that an upregulation of these genes might arise upon transdifferentiation of the cells. In order to prove this hypothesis we performed gene expression analysis on sorted *in vivo*-generated Th1 and Th17 cells cultured for 5 days under neutral- (Th0), Th1-, Th2- or Th17-inducing conditions. Th1 cells showed no differences between HC and RA in cytokine expression except for IFNγ expression in Th1 cells cultured further under Th1-inducing conditions, with higher expression in RA compared to HC (Figure 11B, left panel). This result was not surprising considering that in previous experiments a trend towards higher frequency of total INFγ producing cells and higher frequency of INFγ/IL-17 double producing cells were detected in Th1 cells cultured under Th1 inducing conditions (data not shown). This result was mirrored by a higher TBX21 expression in RA, within the same cells (Figure 11B, right panel). Surprisingly, a trend for higher GATA3 expression in RA, was also found in these cells, particularly under Th2-inducing conditions (Figure 11C, right panel). However, the most interesting result was a consistent trend, for higher RORC expression in RA compared to HC, spanning Th1 cells cultured under all inducing conditions, which was particularly evident under Th1- and Th17-inducing conditions (Figure 11A, right panel).

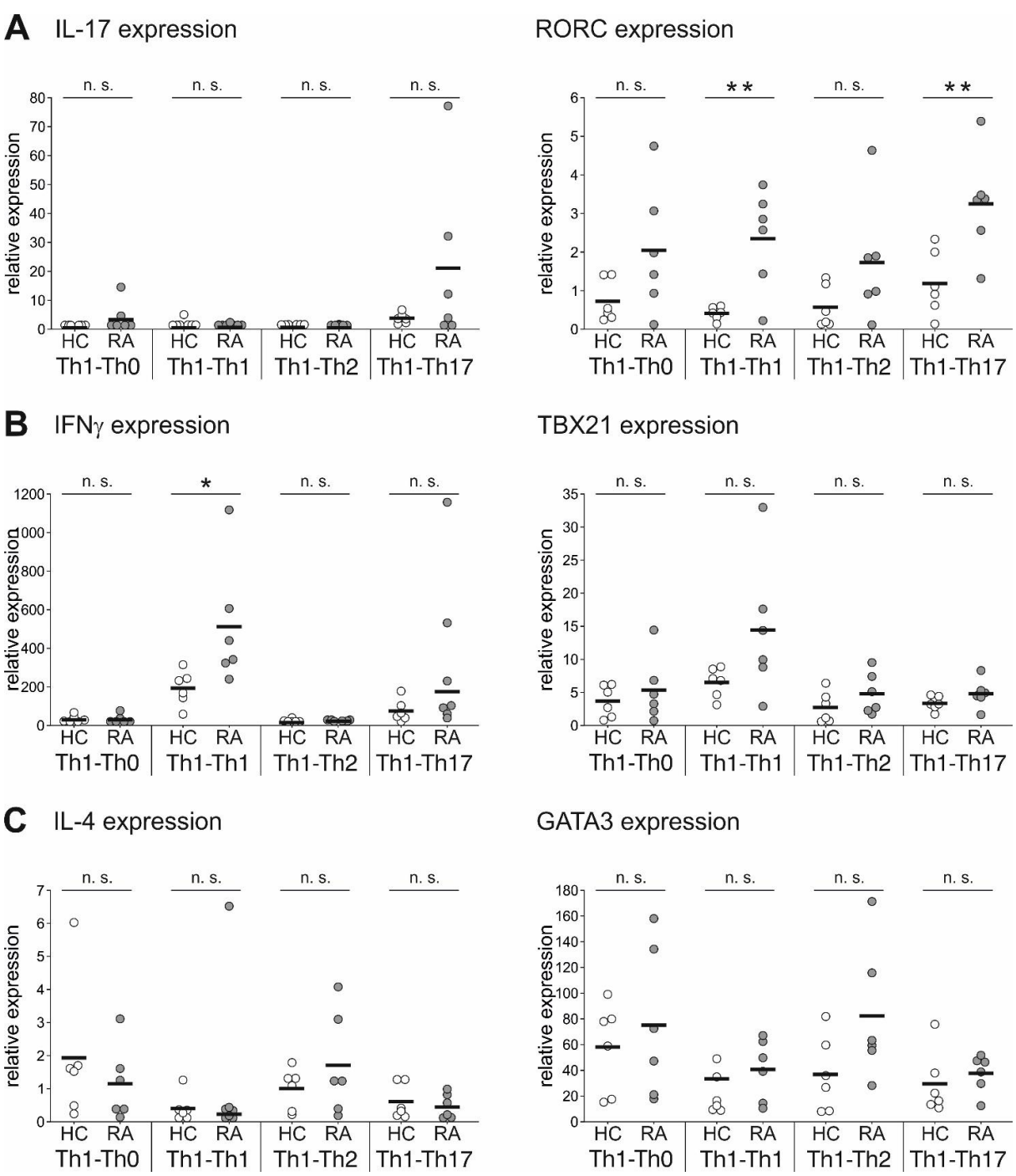


Figure 11. Expression of transcription factors in *in vivo*-derived Th1 cells, transdifferentiated under different inducing conditions. Relative expression of cytokine and transcription factor-encoding genes in Th1 cells cultured for 5 days under Th0-, Th1-, Th2-, and Th17 inducing conditions, assessed by qPCR. (A) IL-17 (left panel) and RORC (right panel), (B) IFNγ and TBX21, (C) IL-4 and GATA3. Healthy individuals (HC, n=4), compared to patients with rheumatoid arthritis (RA, n=3-5).

Next, we performed the same analysis on sorted Th17 cells cultured under the different inducing conditions. No statistically significant differences were detected between HC and

RA in the gene expression of IL-4 and IL-17 (Figure 12A and 12C, left panels). Unsurprisingly, a definitive trend was detectable with lower IFNγ expression levels in RA compared to HC under all inducing conditions, which was particularly evident under Th2-conditions, where it reached statistical significance (Figure 12B, left panel). This phenomenon was also mirrored by the expression levels of TBX21, particularly in cells cultured under neutral and Th1-inducing conditions (Figure 12B, right panel). Surprisingly, under neutral- and Th2-inducing conditions RORC was expressed at lower levels in RA compared to HC, although these differences did not reach statistical significance. On the other hand, Th17 cells cultured under Th1-inducing conditions expressed significantly higher levels of RORC in RA, compared to HC (Figure 12A, right panel). Taken together, these results indicate that the increased plasticity towards the Th17 phenotype of non-Th17 cells in RA, and the resistance of Th17 cells to shift their phenotype towards other Th cell subsets in RA, might be due to an enhanced expression of RORC upon re-stimulation in Th1 cells from RA, and a decreased TBX21 expression in Th17 cells from RA patients, compared to HC.

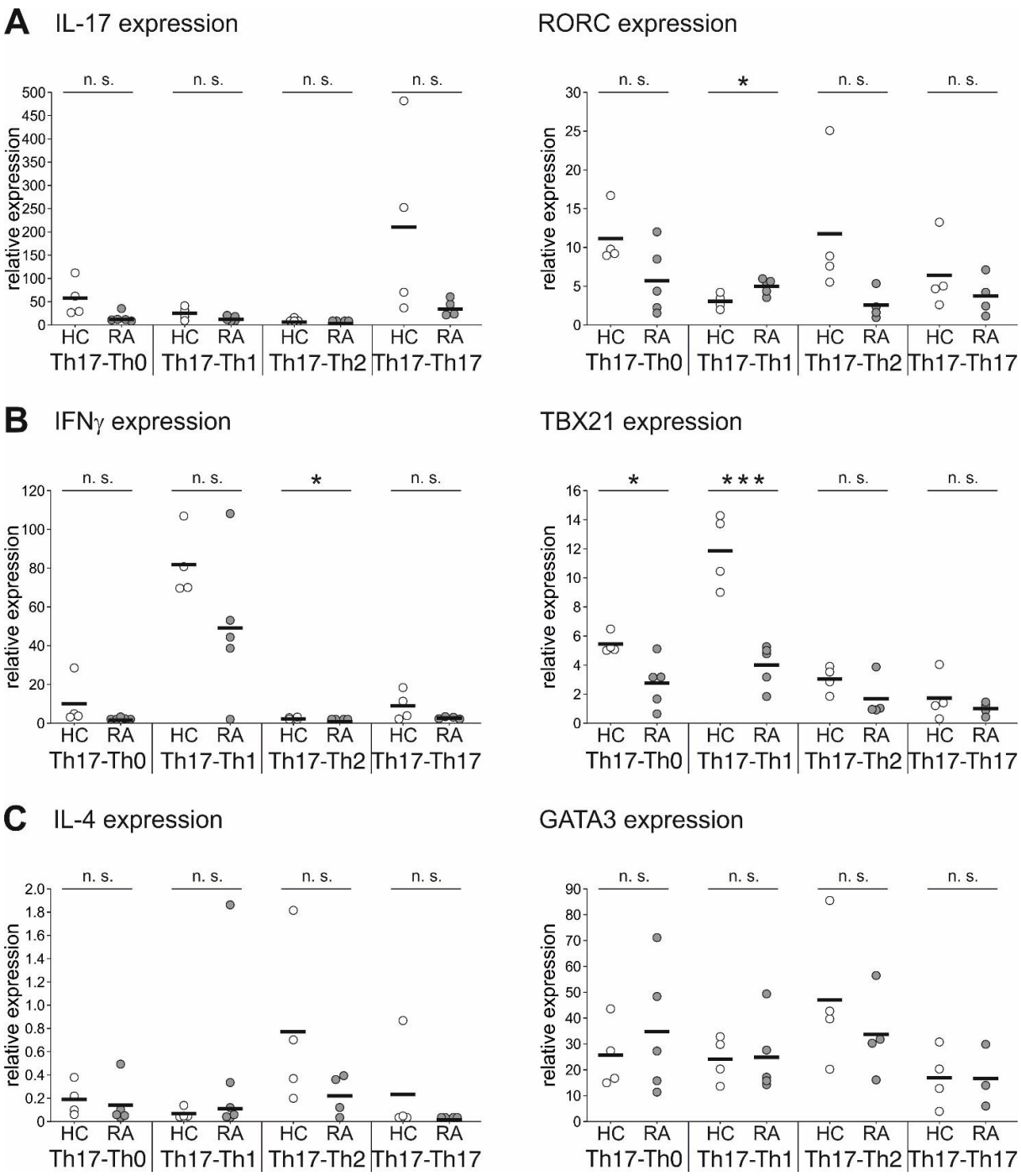


Figure 12. Expression of transcription factors in *in vivo*-derived Th17 cells, transdifferentiated under different inducing conditions. Relative expression of cytokine and transcription factor-encoding genes in Th17 cells cultured for 5 days under Th0-, Th1-, Th2-, and Th17 inducing conditions, assessed by qPCR. (A) IL-17 (left panel) and RORC (right panel), (B) IFNγ and TBX21, (C) IL-4 and GATA3. Healthy individuals (HC: n= 4), compared to patients with rheumatoid arthritis (RA: n=3-5).

3.2.1.2 No differences in histone modifications at key transcription factor loci

Having detected differences in the expression levels of the key transcription factors of Th1 and Th17 cells, potentially underlying the observed altered plasticity of RA, we decided to perform analysis of another level of transcriptional regulation, histone modifications. To this end, we performed chromatin immunoprecipitation, on sorted *in vivo*-generated Th1 and Th17 cells from HC and RA patients, to detect modifications of histone 3 lysine 4 (H3K4), histone 3 lysine 27 (H3K27), and pan-acetylation of histone 3 (H3Ac). We focused our attention on TBX21 and RORC gene loci, however we extended the analysis to include INFG and IL17 gene loci (data not shown). The data consist of the ratio between permissive H3K4, or H3Ac, and repressive H3K27 modifications. Surprisingly, all ratios were positive, indicating a prevalently open chromatin configuration of both gene loci, in both Th1 and Th17. Moreover, there were no significant differences in the ratios of histone modifications in cells derived from both HC and RA patients (Figure 13A and B).

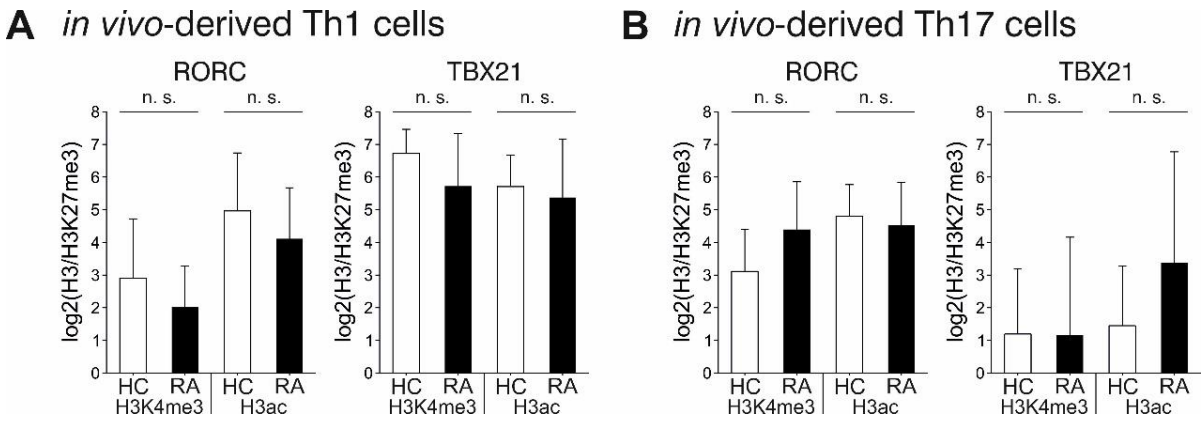


Figure 13. Epigenetic regulation of transcription factors in *in vitro*- and *in vivo*-derived Th1 and Th17 cells. ChIP was performed to analyze enrichment of H3K4me3, H3ac and H3K27me3 at RORC and T-bet promoters. The mean ratios for H3K4me3 and H3ac over H3K27me3 for individual experiments for sorted (A) Th1 cells in HC (n=6) and for RA (n=6), and for sorted (B) Th17 cells in HC (n=5) and RA (n=4) are shown.

3.2.1.3 Enhanced SGK1-Foxo1-IL23R axis in RA Th1 and Th17 cells

Beyond the expression of key transcription factors, several other pathways can influence lineage commitment and plasticity. Among these is the pathway composed of SGK1, FOXO1, and IL23R. The salt-sensing kinase SGK1 is an important indirect inducer of IL23R expression, which is essential for Th17 cell commitment. This induction is mediated by the

degradation of FOXO1, prompted by SGK1, a repressor of IL23R expression. In order to assess a possible role for the elements of this pathway in the differences in plasticity between Th1 and Th17 cells originating from HC and RA, we performed gene expression analysis of these gene loci in sorted, *in vivo*-generated Th1 and Th17 cells. Furthermore, given the previous results for TBX21 and RORC, we also performed the same analysis on Th1 and Th17 cells after culture under opposing inducing conditions. Freshly sorted *in vivo*-generated Th1 cells from RA patients, demonstrated a significantly higher mRNA expression for both SGK1 and IL23R, compared to HCs. More specifically, SGK1 expression was, on average, more than 4 times higher in RA, and IL23R more than 5 times higher in RA, compared to HC. No significant differences in FOXO1 expression were detected (Figure 14A, left panel). Similarly to Th1 cells, also *in vivo*-generated Th17 cells presented a similar pattern of differences between HC and RA. In fact, the difference in SGK1 expression was even greater, with an almost nine fold increase in SGK1 expression in RA compared to HC. IL23R expression was also higher in Th17 cells derived from RA compared to HC. Also in this case, no differences were detected in FOXO1 expression in Th17 cells from HC and RA (Figure 14A, right panel).

Higher gene expression of SGK1 and IL-23R in Th1 cells, from RA patients compared to HC, was also observed after transdifferentiation of these cells under all (neutral, Th1, Th2, Th17) inducing conditions, reaching statistical significance under Th2- and Th17-inducing conditions for SGK1, and Th0-, Th1-, and Th17- inducing conditions for IL23R (Figure 14B, left and right panels). Of note, Th1 cells cultured under Th17 inducing conditions additionally presented a reduced FOXO1 expression in RA (Figure 14B, center). This result is consistent with the fact that, additionally to causing FOXO1 protein degradation, SGK1 activity is also known to reduce FOXO1 transcription. Similarly to transdifferentiated Th1 cells, also Th17 cells showed a trend for higher SGK1 expression in RA compared to HC, under all inducing conditions, and significantly under the opposing Th1-inducing conditions (Figure 14C, left panel). Also IL23R showed a similar trend, achieving statistical significance in cells cultured under Th1-inducing conditions (Figure 14C, right panel). Contrary to Th1 cells, transdifferentiating Th17 cells did not show any significant differences in FOXO1 expression, between cells derived from HC and RA patients (Figure 14C, center). Taken together, these data show an enhanced SGK1-Foxo1-IL23R axis in RA Th1 and Th17 cells, characteristic of the *in* vivo-generated cells, which is then maintained throughout transdifferentiation. This enhanced pathway might contribute to the differential plastic capabilities of Th cells from HC and RA, and ultimately favor the Th17 phenotype in RA.

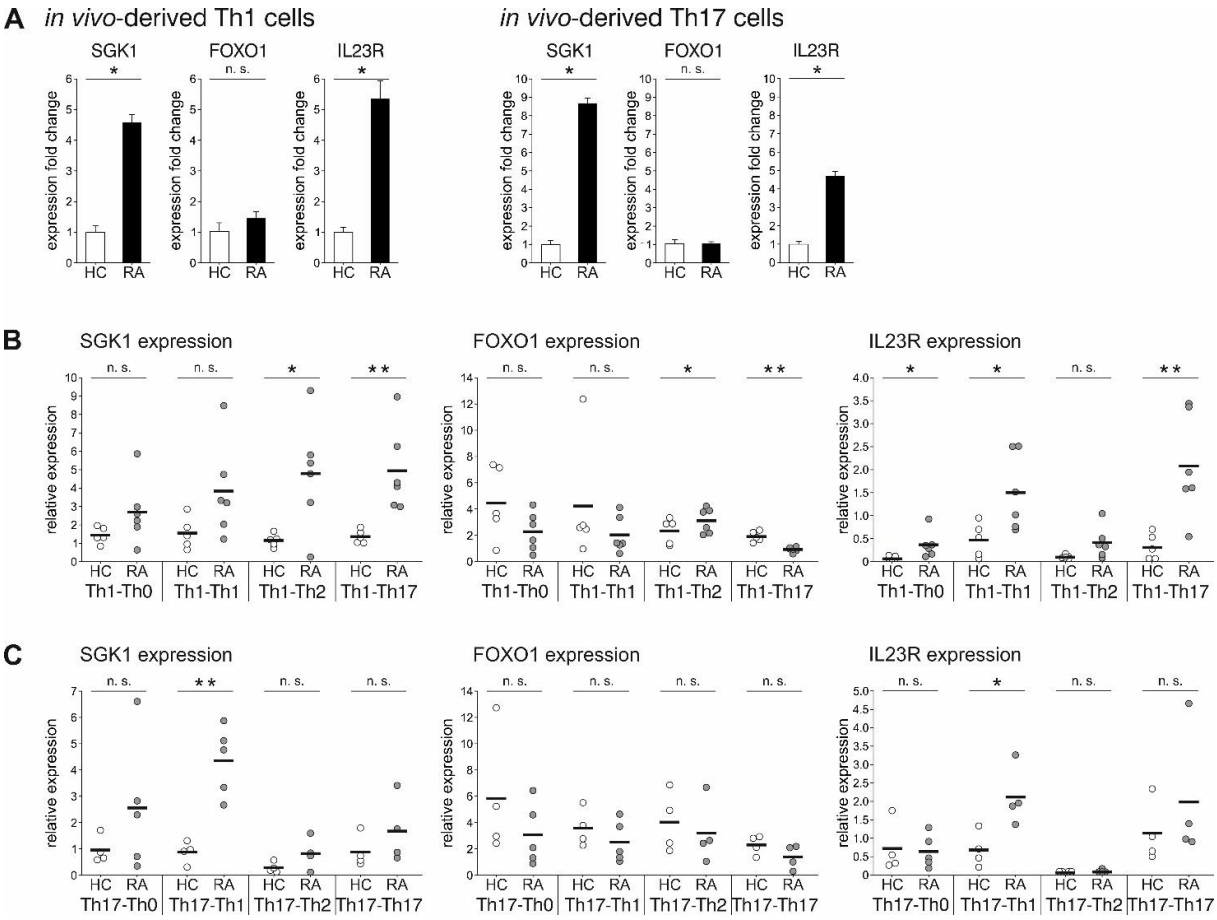


Figure 14. Expression of signalling molecules in *in vivo*-derived sorted Th1 and Th17 cells, and transdifferentiated cells. Relative expression of SGK1, FOXO1 and IL23R assessed by qPCR in (A) *in-vivo* derived Th1 cells and *in-vivo* derived Th17 cells in healthy individuals (HC Th1: n= 5-8, Th17: n=3-5) and patients with rheumatoid arthritis (RA, Th1: n=9-10, Th17: n= 5). Relative expression of SGK1, FOXO1 and IL23R of (B) *in-vivo* derived Th1 cells after transdifferentiation under Th0-, Th1-, Th2-, and Th17-inducing conditions for 5 d, and of (B) *in-vivo* derived Th17 cells after transdifferentiation under Th0-, Th1-, Th2-, and Th17-inducing conditions for 5 d.

4 DISCUSSION

4.1 Plasticity of human *in vitro*- and *in vivo*-generated Th cells

The analysis of the data, that have been presented in the previous sections, allow us to discuss some points. Due to the challenges associated with the tracking of Th cell fate, data on T cell plasticity of human Th cells are scarce. Moreover, the role of T cell plasticity in the pathogenesis of rheumatoid arthritis has not been addressed so far. In this study we demonstrated plasticity from *in vitro*- and *in vivo*-derived Th cells, which was partially not described before and, more importantly, an altered plasticity of *in viv*o-generated Th cells from RA patients.

4.1.1 Th1 cell plasticity

A few studies described Th1 cell plasticity, both in vitro and in vivo, particularly towards the Th2 phenotype (Löhning et al., 2008; Panzer et al., 2012). However, publications on Th1 to Th17 cell conversion are particularly scarce. Tang and collegues reported this kind of plasticity in mouse upon knockout of the oncoprotein and transcriptional regulator Bcl3. However, in the same study, wild type Th1 cells demonstrated a high degree of stability (Tang et al., 2014). Liu et al. have observed a transition of Th1 cells towards a mixed Th1/Th17 phenotype and even a total conversion to Th17 cells. They demonstrated that such shift was induced, in addition to IL-6 and IL-23, by TGFβ, which in turn upregulated Runx1, a transcription factor involved in Th17 differentiation that could both directly bind the IL-17 promoter or induce IL-17 expression indirectly via induction of RORγt (Zhang et al., 2008; Liu et al., 2015). Similar findings were independently published by Brown et al. In this study they also showed a critical role for retinoic acid in repressing Runx1, thereby preventing the skewing of cells towards a Th17 phenotype during Th1 cell development (Brown et al., 2015). Nevertheless, for a very long time both Th1 and Th2 cells have been considered to be very stable after repeated stimulation and several rounds of cell division (Richter et al., 1999). Similarly to the reports on the stability of Th1 cells, in our own experiments on plasticity most Th1 cells, both *in vitro*- and *in vivo*-generated, maintained their phenotype with only very few cells shifting towards other Th cell subtypes such as IFNγ-IL-17+, Th17 cells. However, we observed a considerable plastic capability by *in vitro*-, as well as *in vivo*-generated Th1 cells, to acquire a mixed IFNγ+IL-17+ (Th1/Th17) phenotype. This shift was observed also in Th1 cell cultured under non-Th17 inducing conditions such as Th0- (≈8-9%),

Th1- (≈4-8%), and Th2 inducing conditions (≈10%) (Figures 6 and 8). These results were not previously reported.

A few studies have attempted to describe Th1 plasticity towards the Th2 phenotype. Löhning et al., have demonstrated a low level of plasticity of murine *in vitro*-enriched, LCMV-specific, Th1 cells towards a Th2 phenotype and IFNγ+IL-4+ phenotype 60 days after *in vivo* transfer (0.4 and 2.4%, respectively) (Löhning et al., 2008). Panzer et al., also showed higher but still quite limited *in vivo* plasticity, towards the IL-4 producing phenotype, of *in vitro*-generated Th1 cells upon helminth infection (IFNγ-IL-4+: 4.1% and IFNγ+IL-4+: 9.8%) (Panzer et al., 2012). In our experiments we found a limited degree of plasticity from *in vitro* and *in vivo*-generated Th1 cells towards the IFNγ-IL-4+ (Th2) phenotype (≈1.5%), but a rather higher plasticity towards IFNγ+IL-4+ cells (≈13-24%). Again we observed a significant portion of cells shifting their phenotype towards the expression of IL-17, either alone or while co-expressing IFNγ. This skewing towards the IL-17-producing phenotype was different between *in vivo*-derived Th1 cells from HC and RA, with higher frequencies of both IFNγ-IL-17+ and IFNγ+IL-17+ cells in RA. This result was not previously reported in literature.

The plasticity of Th1 cells, particularly towards the Th1/Th17 phenotype, was further enhanced under Th17-inducing conditions (≈13-30%), in stark contrast to previous findings, described by other groups, in which murine Th1-enriched populations cultured under Th17-inducing condition yielded virtually no IFNγ+IL-17+ cells (Tang et al., 2014). Such a sheer difference might be due to the diverse origin of Th cells and the different inducing conditions applied. Once again we observed an increased tendency of *in vivo*-generated Th1 to transdifferentiate towards an IFNγ-IL17+ phenotype in RA compared to HC, mirrored by lower frequencies of IFNγ+IL17- cells in RA.

In summary, Th1 derived from RA patients showed an enhanced tendency to acquire IL-17 production or even to lose IFNγ production, thereby completely shifting to a Th17 phenotype, compared to the healthy counterparts. Surprisingly, such difference was present even when the Th1 cells were cultured under non-Th17 inducing conditions (namely Th0, Th1, Th2 and Th9 inducing conditions).

4.1.2 Th2 cell plasticity

Several studies have demonstrated a substantial Th2 cell plasticity towards a Th1 phenotype. For example LCMV-specific CD4 cells cultured *in vitro* under Th2-inducing conditions, sorted based on their production of IL-4, and subsequently transferred into LCMV-infected

mice resulted in the majority of the cells completely changing their phenotype to resemble Th1 cells (IFNγ+IL-4- cells: 53.4%) or at least acquiring a mixed phenotype (IFN+IL-4+: 20.7%) (Löhning et al., 2008). In another study, peptide specific Th2 cells were generated and transferred in Listeria-infected mice. In this case 13.9% of Th2 cells converted completely to an IFNγ+IL-4-, Th1 phenotype, while 4.3% acquired IFNγ production in addition to IL-4 (IFNγ+IL-4+) (Krawczyk et al., 2007). A similar shift towards Th1 cells, was achieved when murine Th2 cells were cultured for 6 days under Th1-inducing conditions, with as many as 17% of the cells converting to Th1 cells (IFNγ+IL-4-) and up to 20% of Th2 cells becoming capable of producing both IFNγ and IL-4 (Adeeku et al., 2008). In the same study, previously cited, by Löhning and colleagues, in addition to plasticity of Th1 cells towards the Th2 and Th17 phenotype and of Th2 cells towards the Th1 phenotype, they also investigated the plasticity of Th2 cells towards a Th17 phenotype. As a matter of fact, LCMV-specific Th2 enriched populations gave rise to very low proportions of IL-17 producing cells (≈1.2%), 60 days after transfer in mice infected with LCMV for 30 days (Löhning et al., 2008). We observed a slightly higher plasticity towards IL4+IL-17+ cells for both *in vitro*- and *in vivo*-generated Th2 cells (2-5.5%; Figure 6F and 8F). Moreover, similarly to the results obtained with *in vivo*-generated Th1 cells, also *in vivo*-generated Th2 cells derived from RA patients showed an enhanced tendency to acquire IL-17 production, compared to those derived from HC (Figure 8F). An influence by technical reasons, possibly leading to this result, cannot be excluded since for these experiments Th2 cells were sorted as IL-4 positive and IFNγ negative, without negatively sorting for IL-17. However, prior to the cell sorting, the frequency of cells positive for both IL-4 and IL-17, and at the same time IFNγ negative and IL-17 positive, was extremely low (less than 0.1 percent). Based on this fact we deemed a potential error in the results due to the contaminating outgrowth by these cells to be highly unlikely, especially in light of the observed proliferation rate of T cells, which double every 72 hours upon activation.

Together, these results show a tendency of RA Th subtypes to shift their phenotype towards the Th17 phenotype, possibly contributing to the increased frequencies of Th17 cells, previously observed in the disease. This phenomenon has not been, to our knowledge, previously described.

4.1.3 Th17 cell plasticity

Plasticity of murine Th17 cells has been previously reported by several groups. Regarding plasticity of Th17 cells towards a Th1 phenotype, interesting results were reported by Lexberg

and colleagues. In their study FACS-sorted IL-17 positive cells generated *in vitro* (1 week under Th17 inducing conditions) were cultured for 6 days under Th1 inducing conditions (IL-12 and αIL-4). Their results showed a strong plasticity towards the Th1 phenotype, with up to 66.6% of the cells fully converting to the IFNγ+IL-17-, Th1 phenotype. They replicated this experiment with sorted *in vivo*-generated Th17 cells but surprisingly obtained a much lower frequency (5.9%) of Th1 cells with this experimental setup, while 66.3% maintained the Th17 phenotype and only 8.6% of the cells were capable of producing both cytokines (Lexberg et al., 2008). In a later publication by the same authors they demonstrated a synergic effect of IFNγ and IL-12 in the conversion of *in vivo*-generated Th17 cells into IFNγ+IL-17+, Th1/Th17 cells (Lexberg et al., 2010). Similar results were obtained by Lee et al. (2009), where FACS-sorted Il17f$^{Thy1.1/Thy1.1}$ cells were cultured under Th17 inducing conditions for 6 days, then Thy1.1+ cells were magnetically isolated and cultured for a second round with IL-12. The authors observed a complete shift to the Th1 phenotype for 42.9% of the cells, while 15.9% became capable of producing IFNγ in addition to IL-17 and only 11.2% were resistant to transdifferentiation and maintained their Th17 phenotype (Lee et al., 2009). Comparable results, albeit with slightly different percentages, particularly regarding IFNγ+IL-17+, were obtained by other groups working with murine cells. Such deviations are likely due to differences in the origin and isolation of the cells, and different culture protocols (Kurschus et al., 2010; Thomas et al., 2012).

Data on the plasticity of human Th17 cells is scarce compared to the murine counterpart, even more so in the context of arthritis. However, a few studies have attempted to this phenomenon in the human system. Cohen et al. (2011) have demonstrated a substantial plasticity by *in vivo*-generated Th17 cells, which were FACS-sorted and cultured under neutral or Th1-inducing conditions. While most cells under neutral inducing conditions maintained their Th17 phenotype, under Th1 inducing conditions the majority of them either acquired IFNγ expression in addition to IL-17 (15.5%) or completely shifted to a Th1 phenotype (34.9%) (Cohen et al., 2011). A substantial shift towards the Th1 phenotype was also obtained by Cosmi and colleagues when sorted human Th17 cells were cultured in the presence of IL-12 or, interestingly, synovial fluid of JIA patients (IFNγ+IL-17-: ≈20-24%, IFNγ+IL-17+: ≈30-37%) (Cosmi et al., 2011). In line with these results, in our experiments we found, for both in *vitro* and in *vivo*-derived Th17 cells, a high plastic capability, under Th1 inducing conditions, towards the IFNγ-IL-17+ phenotype (*in vitro*-generated: ≈12-25%, *in vivo*-generated ≈10-20%, Figure 7A and 9A, left column) and IFNγ+IL-17+ cells (*in vitro*-generated: ≈60%, *in vivo*-generated ≈50%, Figure 7A and 9A, right column). Differences to murine *ex vivo* Th17

cells might be based on different intrinsic properties, other experimental setup or different *in vivo* priming making cells more susceptible to Th1-polarizing cytokines, which finally results in higher plasticity of human *in vivo*-derived Th17 cells.

Compared to the plasticity of Th17 cells towards the Th1 phenotype, even fewer studies have explored the transdifferentiation of Th17 cells into Th2 cells. Among these is the already cited study by Lexberg et al. (2008). In addition to culturing *in vivo*-generated Th17 cells under Th1 inducing conditions, they also cultured them under Th2 inducing conditions (IL-4, α-IFNγ, α-IL-12) for 6 days, obtaining only a slight shift towards the Th2 phenotype (IL-4+IL17-: 2.4%, IL-4+IL17+: 1.7%) (Lexberg et al., 2008). Similarly, also in our experiments we obtained only ≈2% of cells converting to each phenotype (Figure 7B and 9B, upper panel, right column).

Also in this case *in vivo*-generated Th17 cells, derived from either HC or RA, behaved differently when cultured under Th1 or Th2 inducing conditions. In detail, while under Th1 inducing conditions the majority of cells from both HC and RA were able to shift to an IFNγ+IL-17+, Th1/Th17 phenotype, and up to 20% were able to totally convert to IFNγ+IL-17- Th1 cells, Th17 cells derived from RA showed a resistance towards this shift with, on average, only 10% of the cells converting to Th1 cells and significantly higher frequencies of cells maintaining an IFNγ-IL-17+ Th17 phenotype, compared to HC. For Th17 cells cultured under Th2 inducing conditions, significantly less cells converted to an IL-4+IL-17- phenotype in RA compared to HC, confirming the increased stability of RA Th17 cells. This increased stability might also contribute to the prominence of the Th17 phenotype observed in the disease. Also this phenomenon has not been, to our knowledge, previously described by other research groups. Together these results suggest that an altered plasticity might contribute to the prominence of Th17 cells observed in early RA.

4.2 Expression levels of factors involved in lineage commitment

4.2.1 Expression levels of the master transcription factors RORC and Tbet

Having observed differences between HC and RA in the plastic capability of *in vivo*-generated Th cells we investigated the possible reasons for this phenomenon, especially considering that at the time of the FACS sorting the cells showed similar phenotypical characteristics in terms of cytokine production. As a first step in this direction, we analyzed factors that might contribute to such difference, in particular the mRNA expression levels of

the master transcription factors of Th17 and Th1 cells RORC and T-bet, in both freshly sorted *in vivo*-generated cells and after transdifferentiation. In addition, mRNA expression for the cytokines IFNγ, IL-4 and IL-17, and for the transcription factor GATA3, were also measured. No differences were found, between RA and HC, in the level of expression of RORC and T-bet, as well as the corresponding cytokines, analyzed in freshly sorted *ex vivo* Th1 and Th17 cells (Figure 10). However, since the populations were sorted based on high expression of IFNγ or IL-17 respectively into pure Th1 and Th17 cells in both HC and RA, the lacking difference is to be expected. Nonetheless, following transdifferentiation of Th1 cells towards Th17 and non-Th17 subtypes and vice-versa, we observed differences between HC and RA. In fact, Th1 cells from RA patients showed a tendency to express higher levels of RORC, upon transdifferentiation, compared to cells derived from healthy individuals. Interestingly, this tendency manifested independently from the inducing conditions, thereby confirming the results previously obtained at the protein level (Figure 11). Also in accordance with the data obtained from the intracellular cytokine stainings, Th17 cells transdifferentiated under Th1 inducing conditions showed a higher expression of RORC in RA, while the expression levels of TBX21 were overall lower in RA compared to HC (Figure 12). Taken together these results confirm the enhanced plastic potential of RA Th1 cells and the increased resistance of Th17 cells derived from RA to transdifferentiate.

4.2.2 Expression levels of elements of the SGK1-FOXO1-IL23R pathway

The importance of the master transcription factors expression is undisputable. Their role is, however, not sufficient to fully explain the intricacies of effector T cell generation and function. Recent studies revealed the complex regulatory network that orchestrates the lineage commitment of Th17 cells, and implied an important role for the salt-sensing kinase SGK1 in Th17 cell generation and stability. Two distinct publications were published in Nature in 2013, focussing in particular on the role of the SGK1 axis. Kleinewietfeld and collegues demonstrated *in vitro* that sodium chloride (NaCl) promotes the stable induction of Th17 cells. Such induction was dependent on SGK1 and characterized by the expression of genes, such as IL-17A, IL-2, and TNFα, associated with a pathogenic phenotype. Moreover, they showed that a high salt diet induced Th17 cells *in vivo* and aggravated EAE. (Kleinewietfeld et al., 2013). Wu and colleagues confirmed the importance of NaCl in SGK1 upregulation and Th17 lineage commitment. Furthermore, rather than focussing solely on SGK1, they explored the entire SGK1-FOXO1-IL23R axis. In their study they demonstrated an induction of SGK1 expression by IL-23 signaling, which in turn phosphorylates FOXO1, a repressor of the *Il23r*

gene, essential for Th17 cell commitment and stability. This modification impedes FOXO1 translocation to the nucleus and mediates its degradation. Since FOXO1 is able to promote its own expression, by binding a site 1kb upstream of the first exon of the *Foxo1* gene locus, SGK1 inhibited not only FOXO1 activity but also its expression and, indirectly, promoted IL23R expression (Essaghir et al., 2009; Wu et al., 2013). In accordance with these results we observed increased expression of SGK1 and IL23R in freshly sorted *ex vivo* RA Th1 and Th17 cells (Figure 14A, left and right panels) as well as after transdifferentiation under opposing conditions (Figure 14B and C), compared to HC. Interestingly FOXO1 was expressed at the same low levels in both freshly sorted Th1 and Th17 cells from HC and RA patients (Figure 14A, left and right panels). Only upon transdifferentiation of Th1 cells under Th17 inducing conditions, the cells derived from RA patients showed a decreased expression of FOXO1, in accordance with the fact that the higher SGK1 expression impedes FOXO1 self-promotion (Figure 14B, central panel). The higher expression of SGK1 might be an intrinsic property of RA Th1 and Th17 cells or, alternatively, it might be linked to a high salt intake, which was previously reported as a possible environmental factor to which RA patients are more frequently exposed (Salgado et al., 2015; Sundström et al., 2015). In light of these results it is worth considering that, while no substantial differences between HC and RA were detected for RORC and T-bet expression in *ex vivo* Th1 and Th17 cells, RORC expression was significantly increased in RA Th1 and Th17 cells after transdifferentiation under Th17-inducing conditions compared to HC, which parallels the shift towards the Th17 phenotype (Figure 11 A and 12 compared to Figure 8 and 9). A possible explanation for this phenomenon could be that RA Th cells are predisposed to a more intense response to Th17-inducing cytokines such as IL-23, for which we observed higher expression of its receptor, as well as SGK1 which is known to be involved in IL23R expression. Together these results indicate that differences in the level of expression of components of the SGK1-FOXO1-IL23R axis, might contribute to the altered plasticity of Th1 and Th17 cells observed in RA, and ultimately to the prevalence of Th17 cells observed in the disease.

4.3 Histone modifications at key transcription factor loci

Whereas fibroblast-like synoviocytes have been extensively studied in RA, data on epigenetic modifications in T cells subpopulations are lacking (Klein et al., 2012). Since epigenetic processes are involved in T cell lineage commitment and controlling gene expression we studied histone modifications at the transcription factor loci RORC and Tbet, in Th1 and Th17 cells of *in vivo*-derived RA and HC. We focused on the permissive trimethylation of

lysine 4 and acetylation of histone 3, which are associated with an opening of the chromatin structure that renders the DNA sequence accessible to transcription factors, and the repressive tri-methylation of lysine 27, which determines a condensation of the chromatin thereby preventing the transcriptional machinery from accessing the underlying DNA sequences. We found the ratio of permissive H3K4me3 and H3Ac to the repressive H3K27me3 to be leaning towards an open chromatin pattern for both RORC and Tbet in both Th1 and Th17 cells. This result is in accordance with our gene expression data. It also resembled the results obtained for TBX21 by Cohen et al., where the gene was characterized by mostly permissive modifications across all sorted T cell subtypes. However, in their study they found a slight prevalence of repressive H3K27me3 at the RORC2 gene in Th1 cells, while their Th0 cells (CD25-CD45RO-CD4+) were characterized by bivalent, both permissive and repressive, modifications which have been associated with genes poised for expression (Azuara et al., 2006; Bernstein et al., 2006). These contrasting results could be attributed to the different sorting strategy, which was based on surface markers rather than cytokine secretion assay. Moreover, in these experiments the cells were expanded for two weeks post sorting and prior to the chromatin immunoprecipitation while we analyzed freshly sorted cells. In our analysis on histone modifications, which were assessed for the first time in *ex vivo*-sorted Th1 and Th17 cells from RA patients and compared to controls, we found no differences. This suggests that the pattern of histone modifications is similar in homogeneous purely isolated Th1 and Th17 cells independently of their HC or RA origin, and that the altered plasticity of RA *in vivo*-generated Th cells seems not to be imprinted at the epigenetic level. Nevertheless, our mRNA expression data on cells transdifferentiated under different inducing conditions seem to indicate an upregulation of RORC and downregulation of TBX21 in RA compared to HC. This suggests that differences in at the histone modifications level, between HC and RA, might arise upon repeated stimulations and in an environment characterized by a cytokine milieu which encourages a shift of these cells towards a different phenotype. Such a possibility might constitute the starting point for further experiments, perhaps involving the epigenetic analysis, both at the histone- and DNA-level, of sorted of IFNγ-IL-17+ and IFNγ+IL-17- populations after transdifferentiation from *in vivo*-generated Th1 and Th17 cells, from HC and RA, respectively. Furthermore, the lack of differences between HC and RA in freshly sorted *in vivo*-generated Th1 cells, both at the epigenetic and gene expression levels, could be attributed to the sorting of Th1 cells as a single population. As a matter of fact, in recent years it has been demonstrated that Th1 cells are constituted by two distinct populations, “classic“ and “non-classic“ Th1 cells. Non-classic Th1 cells have been shown to be derived from Th17 cells which lost IL-17 production. They share with Th17 cells the

expression of the surface markers CCR6 and CD161. Differences in frequencies, proliferation or plastic capabilities of these cells between HC and RA might explain the results reported in this thesis and are currently the object of further experiments.

5 CONCLUDING REMARKS

In summary we demonstrated plasticity in both *in vitro*- and *in vivo*-generated human Th cells. We further demonstrated for the first time an altered plasticity of RA *in vivo*-derived-Th cells which manifests itself in the propensity of non-Th17 cells from RA, including but not limited to Th1 cells, to shift their phenotype to resemble an IL-17-producing phenotype, and in the tendency of Th17 cells derived from RA patients to be more resistant to the shift towards non-IL-17-producing phenotypes. These phenomena lead to a predominance of cells belonging to the Th17 phenotype, characteristic for the disease. We expected a strong involvement of the master transcription factors RORC and Tbet in this phenomenon. While we did not observe differences in the levels of expression of these factors between HC- and RA-cells in freshly sorted cells, we reported an increased expression of RORC in both RA-derived Th1 and Th17 cells upon trasdifferentiation, and a decrease in TBX21 in Th1 cells. We further investigated the epigenetic status of these cells but did not find any differences between HC and RA in the histone modifications at the master transcription factor loci. These results suggest that histone modifications, and thereby transcription factor expression regulation, might be involved in the observed phenomenon but only in response to secondary stimulation and culture conditions and, perhaps, in response to environmental factors. Further studies, possibly involving re-sorting of transdifferentiated Th1 and Th17 populations could shed light on the contribution of histone modifications to the altered plasticity observed in RA-derived Th cells. Furthermore, a significant role might be played by other epigenetic modifications such as DNA methylation. Another element that could bring a higher resolution to this yet unclear picture would be the analysis of epigenetic modifications in Th1 and Th17 sub-populations such as “classic” and “non-classic” Th1 cells as well as GM-CSF-producing “pathogenic” and IL-10-producing “non-pathogenic” Th17 cells. In our search for possible contributors to the altered Th plasticity observed in RA we analyzed the expression levels of elements belonging to the SGK1-FOXO1-IL23R axis. We found increased expression levels of SGK1 and IL23R in RA-derived, freshly sorted Th1 and Th17. As IL-23 signaling is fundamental for Th17 lineage commitment and stability, this could provide RA Th1 cells with an initial “advantage”, over HC-derived Th cells, opening a path for transdifferentiation towards the Th17 phenotype. Meanwhile, for the same reason, a higher IL23R expression in RA Th17 cells might give them an enhanced stability compared to the HC counterparts. Since SGK1 expression and activity is enhanced by a high intake of NaCl, it would be of great interest, in future studies to analyze a possible correlation of this environmental factor with Th17 frequency and RA incidence.

Together, these results represent a step forward towards the understanding of the potential role of Th cell plasticity in the pathogenesis of rheumatoid arthritis. Further experiments will be required to pinpoint the epigenetic and molecular mechanisms that are responsible for the altered plasticity observed in RA-derived Th cells.

BIBLIOGRAFY

Adeeku E, Gudapati P, Mendez-Fernandez Y, Van Kaer L, Boothby M. (2008). Flexibility accompanies commitment of memory CD4 lymphocytes derived from IL-4 locus-activated precursors. *Proc Natl Acad Sci U S A.* **105**(27):9307-12.

Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* **8:** 942-9.

Acuto O, Michel F. (2003). CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol.* **3**(12): 939-51.

Aderem A, Underhill DM. (1999). Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol.* **17**: 593-623.

Agarwal S, Rao A. (2003). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity*. **9**(6): 765-75.

Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem.* 278(3): 1910-4.

Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ, Frucht DM. (2003). Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol.* **23**(3): 147-61.

Akimzhanov AM, Yang XO, Dong C. (2007). Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. *J Biol Chem.* **282**(9): 5969-72.

Akira S, Uematsu S, Takeuchi O. (2006). Pathogen recognition and innate immunity. *Cell.* **124**(4): 783-801.

Alamanos Y, Drosos AA. (2005). Epidemiology of adult rheumatoid arthritis. Autoimmun Rev. **4**(3): 130-6.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. (2007). Molecular Biology of the Cell. Lymphocytes and the Cellular Basis of Adaptive Immunity. **5th**, Garland Science, New York.

Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovský J, Wolfe F, Hawker G. (2010). 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* **62**(9): 2569-81.

Alzabin S, Abraham SM, Taher TE, Palfreeman A, Hull D, McNamee K, Jawad A, Pathan E, Kinderlerer A, Taylor PC, Williams R, Mageed R. (2012). Incomplete response of

inflammatory arthritis to TNFα blockade is associated with Th17 pathway. *Ann Rheum Dis*. **71**(10): 1741-8.

Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. (2012). Defining the human T helper 17 cell phenotype. *Trends Immunol.* **33**(10): 505-12.

Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. (2013). Main features of human T helper 17 cells. *Ann N Y Acad Sci.* **1284**: 66-70.

Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Filì L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S. (2007). Phenotypic and functional features of human Th17 cells. *J Exp Med.* **204**(8): 1849-61.

Aramburu J, López-Rodríguez C. (2009). Brx shines a light on the route from hyperosmolarity to NFAT5. *Sci Signal*. **2**(65): pe20.

Awane M, Andres PG, Li DJ, Reinecker HC. (1999). NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol.* **162**(9): 5337-44.

Azuara V, Perry P, Sauer S, Spivakov M, Jørgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merkenschlager M, Fisher AG. (2006). Chromatin signatures of pluripotent cell lines. *Nat Cell Biol.* **8**(5): 532-8.

Bannister AJ, Kouzarides T. (2011). Regulation of chromatin by histone modifications. *Cell Res*. **21**(3): 381-395

Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I, Dinarello CA, Paul WE. (2009). IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A.* **106**(17): 7119-24.

Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441:** 235-8.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell.* **125**(2): 315-26.

Binger KJ, Linker RA, Muller DN, Kleinewietfeld M. (2015). Sodium chloride, SGK1, and Th17 activation. *Pflugers Arch*. **467**(3): 543-50.

Brown CC, Esterhazy D, Sarde A, London M, Pullabhatla V, Osma-Garcia I, Al-Bader R, Ortiz C, Elgueta R, Arno M, de Rinaldis E, Mucida D, Lord GM, Noelle RJ. (2015). Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program. *Immunity.* **42**(3): 499-511.

Brüstle A, Heink S, Huber M, Rosenplänter C, Stadelmann C, Yu P, Arpaia E, Mak TW, Kamradt T, Lohoff M. (2007). The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol*. **8**(9): 958-66.

Buch MH, Boyle DL, Rosengren S, Saleem B, Reece RJ, Rhodes LA, Radjenovic A, English A, Tang H, Vratsanos G, O'Connor P, Firestein GS, Emery P. (2009). Mode of action of abatacept in rheumatoid arthritis patients having failed tumour necrosis factor blockade: a histological, gene expression and dynamic magnetic resonance imaging pilot study. *Ann Rheum Dis.* **68**(7): 1220-27.

Cervoni N, Szyf M. (2001). Demethylase activity is directed by histone acetylation. *J Biol Chem.* **276:** 40778-87.

Chabaud M, Fossiez F, Taupin JL, Miossec P. (1998). Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J Immunol*. **161**(1): 409-14.

Chabaud M, Lubberts E, Joosten L, van Den Berg W, Miossec P. (2001). IL-17 derived from juxta-articular bone and synovium contributes to joint degradation in rheumatoid arthritis. *Arthritis Res*. **3**(3): 168-77.

Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, Tato C, Yoshimura A, Hennighausen L, O'Shea JJ. (2006). Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A.* **103**(21): 8137-8142.

Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q, Dong C. (2009). Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity.* **30**(4): 576-87.

Cohen CJ, Crome SQ, MacDonald KG, Dai EL, Mager DL, Levings MK. (2011). Human Th1 and Th17 cells exhibit epigenetic stability at signature cytokine and transcription factor loci. *J Immunol.* **187**(11): 5615-26.

Cosmi L, Cimaz R, Maggi L, Santarlasci V, Capone M, Borriello F, Frosali F, Querci V, Simonini G, Barra G, Piccinni MP, Liotta F, De Palma R, Maggi E, Romagnani S, Annunziato F. (2011). Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis Rheum.* **63**(8): 2504-15.

Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, Rodolico G, Querci V, Abbate G, Angeli R, Berrino L, Fambrini M, Caproni M, Tonelli F, Lazzeri E, Parronchi P, Liotta F, Maggi E, Romagnani S, Annunziato F. (2008). Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J Exp Med.* **205**(8): 1903-16.

Cosmi L, Maggi L, Santarlasci V, Capone M, Cardilicchia E, Frosali F, Querci V, Angeli R, Matucci A, Fambrini M, Liotta F, Parronchi P, Maggi E, Romagnani S, Annunziato F. (2010). Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol.* **125**(1): 222-30.

Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature.* **421**(6924): 744-8.

Cyster JG. (1999). Chemokines and cell migration in secondary lymphoid organs. *Science.* **286**(5447): 2098-102.

Cyster JG. (2005). Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol.* **23**: 127-59.

de Beaucoudrey L, Puel A, Filipe-Santos O, Cobat A, Ghandil P, Chrabieh M, Feinberg J, von Bernuth H, Samarina A, Jannière L, Fieschi C, Stéphan JL, Boileau C, Lyonnet S, Jondeau G, Cormier-Daire V, Le Merrer M, Hoarau C, Lebranchu Y, Lortholary O, Chandesris MO, Tron F, Gambineri E, Bianchi L, Rodriguez-Gallego C, Zitnik SE, Vasconcelos J, Guedes M, Vitor AB, Marodi L, Chapel H, Reid B, Roifman C, Nadal D, Reichenbach J, Caragol I, Garty BZ, Dogu F, Camcioglu Y, Gülle S, Sanal O, Fischer A, Abel L, Stockinger B, Picard C, Casanova JL. (2008). Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J Exp Med.* **205**(7): 1543-50.

den Broader AA, Joosten LA, Saxne T, Heinegård D, Fenner H, Miltenburg AM, Frasa WL, van Tits LJ, Buurman WA, van Riel PL, van de Putte LB, Barrera P. (2002). Long term anti-tumour necrosis factor alpha monotherapy in rheumatoid arthritis: effect on radiological course and prognostic value of markers of cartilage turnover and endothelial activation. *Ann Rheum Dis*. **61**(4): 311-8.

Dixon WG, Hyrich KL, Watson KD, Lunt M, Galloway J, Ustianowski A; BSRBR Control Centre Consortium, Symmons DP; BSR Biologics Register. (2010). Drug-specific risk of tuberculosis in patients with rheumatoid arthritis treated with anti-TNF therapy: results from the British Society for Rheumatology Biologics Register (BSRBR). *Ann Rheum Dis*. **69**(3): 522-8.

Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. (2009). Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol*. **10**(8): 857-63.

Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, Takahashi H, Sun HW, Kanno Y, Powrie F, O'Shea JJ. (2010). Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity.* **32**(5): 605-15.

Eder AM, Dominguez L, Franke TF, Ashwell JD. (1998). Phosphoinositide 3-kinase regulation of T cell receptor-mediated interleukin-2 gene expression in normal T cells. *J Biol Chem.* **273**(43): 28025-31.

Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg A, Mauri C. (2004). Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. *J Exp Med*. **200**(3): 277-85.

Essaghir A, Dif N, Marbehant CY, Coffer PJ, Demoulin JB. (2009). The transcription of FOXO genes is stimulated by FOXO3 and repressed by growth factors. *J Biol Chem*. **284**(16): 10334-42.

Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorisio T, Traidl-Hoffmann C, Behrendt H, Durham SR, Schmidt-Weber CB, Cavani A. (2009). Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest*. **119**(12): 3573-85.

Eyerich S, Zielinski CE. (2014). Defining Th-cell subsets in a classical and tissue-specific manner: Examples from the skin. *Eur J Immunol*. **44**(12): 3475-83.

Farez MF, Fiol MP, Gaitán MI, Quintana FJ, Correale J. (2014). Sodium intake is associated with increased disease activity in multiple sclerosis. *J Neurol Neurosurg Psychiatry*. **86**(1): 26-31.

Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. (2003). IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J Immunol.* **170**(4): 2106-12.

Fontenot JD, Gavin MA, Rudensky AY. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol.* **4**: 330-6.

Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM. (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity.* **15**(6): 985-95.

Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, Pin JJ, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das Mahapatra B, Rouvier E, Golstein P, Banchereau J, Lebecque S. (1996). T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med.* **183**(6): 2593-603.

Ganz T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol.* **3**(9): 710-20.

Geginat J, Paroni M, Maglie S, Alfen JS, Kastirr I, Gruarin P, De Simone M, Pagani M, Abrignani S. (2014). Plasticity of human CD4 T cell subsets. *Front Immunol*. **5**: 630.

Genovese MC, Braun DK, Erickson JS, Berclaz PY, Banerjee S, Heffernan MP, Carlier H. (2016). Safety and efficacy of open-label subcutaneous ixekizumab treatment for 48 weeks in a phase II study in biologic-naive and TNF-IR Patients with rheumatoid arthritis. *J Rheumatol.* **43**(2): 289-97.

Genovese MC, Durez P, Richards HB, Supronik J, Dokoupilova E, Aelion JA, Lee SH, Codding CE, Kellner H, Ikawa T, Hugot S, Ligozio G, Mpofu S. (2014). One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: phase II, dose-finding, double-blind, randomized, placebo-controlled study. *J Rheumatol*. **41**(3): 414-21.

Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE, Ramos HL, Wei L, Davidson TS, Bouladoux N, Grainger JR, Chen Q, Kanno Y, Watford WT, Sun HW, Eberl G, Shevach EM, Belkaid Y, Cua DJ, Chen W, O'Shea JJ. (2010). Generation of pathogenic T(H)17 cells in the absence of TGF-β signalling. *Nature.* **467**(7318): 967-71.

Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. (2004). Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity.* **21:** 589-601.

Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. (2002). Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol.* **20**: 621-67.

Harris TJ, Grosso JF, Yen HR, Xin H, Kortylewski M, Albesiano E, Hipkiss EL, Getnet D, Goldberg MV, Maris CH, Housseau F, Yu H, Pardoll DM, Drake CG. 2007. Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. *J Immunol.* **179**(7): 4313-7.

Hashimoto H, Vertino PM, Cheng X. (2010). Molecular coupling of DNA methylation and histone methylation. *Epigenomics*. **2**(5): 657-69.

Heikamp EB, Patel CH, Collins S, Waickman A, Oh MH, Sun IH, Illei P, Sharma A, Naray-Fejes-Toth A, Fejes-Toth G, Misra-Sen J, Horton MR, Powell JD. (2014). The AGC kinase SGK1 regulates TH1 and TH2 differentiation downstream of the mTORC2 complex. *Nat Immunol*. **15**(5): 457-64.

Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. (1989). Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp Med*. **169**(1): 59-72.

Hegazy AN, Peine M, Helmstetter C, Panse I, Fröhlich A, Bergthaler A, Flatz L, Pinschewer DD, Radbruch A, Löhning M. (2010). Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity*. **32**(1): 116-28.

Hirahara K, Ghoreschi K, Laurence A, Yang XP, Kanno Y, O'Shea JJ. (2010). Signal transduction pathways and transcriptional regulation in Th17 cell differentiation. *Cytokine Growth Factor Rev.* **21**(6): 425-34.

Hogquist KA, Tomlinson AJ, Kieper WC, McGargill MA, Hart MC, Naylor S, Jameson SC. (1997). Identification of a naturally occurring ligand for thymic positive selection. *Immunity*. **6**(4): 389-99.

Holland SM, DeLeo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N, Freeman AF, Demidowich A, Davis J, Turner ML, Anderson VL, Darnell DN, Welch PA, Kuhns DB, Frucht DM, Malech HL, Gallin JI, Kobayashi SD, Whitney AR, Voyich JM, Musser JM, Woellner C, Schäffer AA, Puck JM, Grimbacher B. (2007). STAT3 mutations in the hyper-IgE syndrome. *N Engl J Med.* **357**(16): 1608-19.

Grunstein M. (1997). Histone acetylation in chromatin structure and transcription. *Nature*. **389**(6649): 349-52.

Honorati MC, Meliconi R, Pulsatelli L, Canè S, Frizziero L, Facchini A. (2001). High in vivo expression of interleukin-17 receptor in synovial endothelial cells and chondrocytes from arthritis patients. *Rheumatology (Oxford)*. **40**(5): 522-7.

Hot A, Miossec P. (2011). Effects of interleukin (IL)-17A and IL-17F in human rheumatoid arthritis synoviocytes. *Ann Rheum Dis*. **70**(5): 727-32.

Hymowitz SG, Filvaroff EH, Yin JP, Lee J, Cai L, Risser P, Maruoka M, Mao W, Foster J, Kelley RF, Pan G, Gurney AL, de Vos AM, Starovasnik MA. (2001). IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* **20**(19): 5332-41.

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* **126**(6): 1121-33.

Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, Takaesu G, Hori S, Yoshimura A, Kobayashi T. (2008). Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *J Biol Chem.* **283**(25): 17003-8.

Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP. (1998). IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol.* **160**(7): 3513-21.

Kane LP, Andres PG, Howland KC, Abbas AK, Weiss A. (2001). Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not TH2 cytokines. *Nat Immunol.* **2**(1): 37-44.

Kanno Y, Vahedi G, Hirahara K, Singleton K, O'Shea JJ. (2012). Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annu Rev Immunol*. **30**: 707-31.

Kaplan MH. (2013). Th9 cells: differentiation and disease. *Immunol Rev*. **252**(1): 104-15.

Kaplan MH, Hufford MM, Olson MR. (2015). The development and in vivo function of T helper 9 cells. *Nat Rev Immunol*. **15**(5): 295-307.

Katz Y, Nadiv O, Beer Y. (2001). Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes. *Arthritis Rheum*. **44**(9): 2176-84.

Kebir H, Ifergan I, Alvarez JI, Bernard M, Poirier J, Arbour N, Duquette P, Prat A. (2009). Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol.* **66**(3): 390-402.

Klein K, Ospelt C, Gay S. (2012). Epigenetic contributions in the development of rheumatoid arthritis. *Arthritis Res Ther*. **14**(6): 227.

Kleinewietfeld M, Manzel A, Titze J, Kvakan H, Yosef N, Linker RA, Muller DN, Hafler DA. (2013). Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature*. **496**(7446): 518-22.

Koenders MI, Lubberts E, van de Loo FA, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Kolls JK, Di Padova FE, Joosten LA, van den Berg WB. (2006). Interleukin-17 acts independently of TNF-alpha under arthritic conditions. *J Immunol*. **176**(10):6262-9.

Korn T, Bettelli E, Oukka M, Kuchroo VK. (2009). IL-17 and Th17 Cells. *Annu Rev Immunol.* **27**: 485-517.

Koshy PJ, Henderson N, Logan C, Life PF, Cawston TE, Rowan AD. (2002). Interleukin 17 induces cartilage collagen breakdown: novel synergistic effects in combination with proinflammatory cytokines. *Ann Rheum Dis*. **61**(8): 704-13.

Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT, Martin TJ, Suda T. (1999). IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest*. **103**(9): 1345-52.

Kotake S, Nanke Y, Yago T, Kawamoto M, Kobashigawa T, Yamanaka H. (2016). Ratio of Circulating IFNγ (+) "Th17 Cells" in Memory Th Cells Is Inversely Correlated with the Titer of Anti-CCP Antibodies in Early-Onset Rheumatoid Arthritis Patients Based on Flow Cytometry Methods of the Human Immunology Project. *Biomed Res Int.* **2016**(2016): 9694289.

Kotenko SV, Izotova LS, Mirochnitchenko OV, Esterova E, Dickensheets H, Donnelly RP, Pestka S. (2001). Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. *J Biol Chem.* **276**(4): 2725-32.

Kouzarides T. (2007). Chromatin modifications and their function. *Cell*. **128**(4): 693-705.

Krawczyk CM, Shen H, Pearce EJ. (2007). Functional plasticity in memory T helper cell responses. *J Immunol*. **178**(7): 4080-8.

Kugyelka R, Kohl Z, Olasz K, Mikecz K, Rauch TA, Glant TT, Boldizsar F. (2016). Enigma of IL-17 and Th17 Cells in Rheumatoid Arthritis and in Autoimmune Animal Models of Arthritis. *Mediators Inflamm*. **2016**:6145810.

Kunwar S, Dahal K, Sharma S. (2016). Anti-IL-17 therapy in treatment of rheumatoid arthritis: a systematic literature review and meta-analysis of randomized controlled trials. *Rheumatol Int*. **36**(8): 1065-75.

Kurschus FC, Croxford AL, Heinen AP, Wörtge S, Ielo D, Waisman A. (2010). Genetic proof for the transient nature of the Th17 phenotype. *Eur J Immunol*. **40**(12): 3336-46.

Laan M, Cui ZH, Hoshino H, Lötvall J, Sjöstrand M, Gruenert DC, Skoogh BE, Lindén A. (1999). Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol.* **162**(4): 2347-52.

Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* **201**(2): 233-40.

Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, Shevach EM, O'shea JJ. (2007). Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity.* **26**(3): 371-81.

Lauritsen JP, Haks MC, Lefebvre JM, Kappes DJ, Wiest DL. (2006). Recent insights into the signals that control alphabeta/gammadelta-lineage fate. *Immunol Rev*. **209**: 176-90.

Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, Weaver CT. (2009). Late developmental plasticity in the T helper 17 lineage. *Immunity*. **30**(1): 92-107.

Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, Skapenko A. (2010). Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum*. **62**(10): 2876-85.

Lexberg MH, Taubner A, Albrecht I, Lepenies I, Richter A, Kamradt T, Radbruch A, Chang HD. (2010). IFN-γ and IL-12 synergize to convert in vivo generated Th17 into Th1/Th17 cells. *Eur J Immunol*. **40**(11): 3017-27.

Lexberg MH, Taubner A, Förster A, Albrecht I, Richter A, Kamradt T, Radbruch A, Chang HD. (2008). Th memory for interleukin-17 expression is stable in vivo. *Eur J Immunol.* **38**(10): 2654-64.

Liu HP, Cao AT, Feng T, Li Q, Zhang W, Yao S, Dann SM, Elson CO, Cong Y. (2015). TGF-β converts Th1 cells into Th17 cells through stimulation of Runx1 expression. *Eur J Immunol.* **45**(4): 1010-8.

Liu SJ, Tsai JP, Shen CR, Sher YP, Hsieh CL, Yeh YC, Chou AH, Chang SR, Hsiao KN, Yu FW, Chen HW. (2007). Induction of a distinct CD8 Tnc17 subset by transforming growth factor-beta and interleukin-6. *J Leukoc Biol.* **82**(2): 354-60.

Lockhart E, Green AM, Flynn JL. (2006). IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol.* **177**(7): 4662-9.

Löhning M, Hegazy AN, Pinschewer DD, Busse D, Lang KS, Höfer T, Radbruch A, Zinkernagel RM, Hengartner H. (2008). Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J Exp Med.* **205**(1):53-61.

Lubberts E, van den Bersselaar L, Oppers-Walgreen B, Schwarzenberger P, Coenen-de Roo CJ, Kolls JK, Joosten LA, van den Berg WB. (2003). IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-kappa B ligand/osteoprotegerin balance. *J Immunol.* **170**(5): 2655-62.

Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, Tangye SG, Cook MC. (2008). Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med.* **205**(7): 1551-7.

Maggi L, Santarlasci V, Capone M, Rossi MC, Querci V, Mazzoni A, Cimaz R, De Palma R, Liotta F, Maggi E, Romagnani S, Cosmi L, Annunziato F. (2012). Distinctive features of classic and nonclassic (Th17 derived) human Th1 cells. *Eur J Immunol.* **42**(12): 3180-8.

Martel-Pelletier J, Mineau F, Jovanovic D, Di Battista JA, Pelletier JP. (1999). Mitogen-activated protein kinase and nuclear factor kappaB together regulate interleukin-17-induced nitric oxide production in human osteoarthritic chondrocytes: possible role of transactivating factor mitogen-activated protein kinase-activated proten kinase (MAPKAPK). *Arthritis Rheum.* **42**(11): 2399-409.

Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, O'Malley JT, Kapur R, Levy DE, Kansas GS, Kaplan MH. (2007). Stat3 and Stat4 direct development of IL-17-secreting Th cells. *J Immunol.* **178**(8): 4901-7.

Mazzoni A, Santarlasci V, Maggi L, Capone M, Rossi MC, Querci V, De Palma R, Chang HD, Thiel A, Cimaz R, Liotta F, Cosmi L, Maggi E, Radbruch A, Romagnani S, Dong J, Annunziato F. (2015). Demethylation of the RORC2 and IL17A in human CD4+ T lymphocytes defines Th17 origin of nonclassic Th1 cells. *J Immunol.* **194**(7): 3116-26.

McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ. (2007). TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol.* **8**(12): 1390-7.

McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, McClanahan TK, O'Shea JJ, Cua DJ. (2009). The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol.* **10**(3): 314-24.

McInnes IB, Schett G. (2011). The pathogenesis of rheumatoid arthritis. *N Engl J Med*. **365**(23): 2205-19.

Mellado M, Martínez-Muñoz L, Cascio G, Lucas P, Pablos JL, Rodríguez-Frade JM. (2015). T Cell Migration in Rheumatoid Arthritis. *Front Immunol*. **6**: 384.

Messi M, Giacchetto I, Nagata K, Lanzavecchia A, Natoli G, Sallusto F. (2003). Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat Immunol.* **4**(1): 78-86.

Mewar D, Wilson AG. (2011). Treatment of rheumatoid arthritis with tumour necrosis factor inhibitors. *Br J Pharmacol*. **162**(4): 785-91.

Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD, Hess JL. (2002). MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell.* **10:** 1107-17.

Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, Kanno Y, Spalding C, Elloumi HZ, Paulson ML, Davis J, Hsu A, Asher AI, O'Shea J, Holland SM, Paul WE, Douek DC. (2008). Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature.* **452**(7188): 773-6.

Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, Kawamura N, Ariga T, Pasic S, Stojkovic O, Metin A, Karasuyama H. (2007). Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature.* **448**(7157): 1058-62.

Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, Olivenstein R, Elias J, Chakir J. (2001). IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol.* **108**(3): 430-8.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* **136**(7): 2348-57.

Mosmann TR, Coffman RL. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. **7**: 145-73.

Mowen KA, Glimcher LH. (2004). Signaling pathways in Th2 development. *Immunol Rev.* **202**: 203-22.

Mukasa R, Balasubramani A, Lee YK, Whitley SK, Weaver BT, Shibata Y, Crawford GE, Hatton RD, Weaver CT. (2010). Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity.* **32**(5): 616-27.

Muranski P and Restifo NP. (2013). Essentials of Th17 cell commitment and plasticity. *Blood.* **121**(13): 2402-14.

Murphy KP. 2012. Janeway's Immunobiology. Garland Science, New York.

Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ. (2003). Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med.* **198**(12): 1951-7.

Nistala K and Weddeburn LR. (2009). Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis. *Rheumatology*. **48**(6): 602-6.

Norton M, Screaton RA. (2014). SGK1: master and commander of the fate of helper T cells. *Nat Immunol*. **15**(5): 411-3.

Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C. (2007). *Nature.* **448**(7152): 480-3.

Ooi SK, Qiu C, Bernstein E, Li K, Jia D, Yang Z, Erdjument-Bromage H, Tempst P, Lin SP, Allis CD et al. (2007). DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature.* **448:** 714-7.

Okamoto S, Fujiwara H, Nishimori H, Matsuoka K, Fujii N, Kondo E, Tanaka T, Yoshimura A, Tanimoto M, Maeda Y. (2015). Anti-IL-12/23 p40 antibody attenuates experimental chronic graft-versus-host disease via suppression of IFN-γ/IL-17-producing cells. *J Immunol*. **194**(3): 1357-63.

Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* **13**(5): 715-25.

Ouyang W, Kolls JK, Zheng Y. (2008). The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity.* **28**(4): 454-67.

Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol.* **8:** 1353-62.

Panzer M, Sitte S, Wirth S, Drexler I, Sparwasser T, Voehringer D. (2012). Rapid in vivo conversion of effector T cells into Th2 cells during helminth infection. *J Immunol.* **188**(2): 615-23.

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunology.* **6**(11): 1133-41.

Pavelka K, Chon Y, Newmark R, Lin SL, Baumgartner S, Erondu N. (2015). A study to evaluate the safety, tolerability, and efficacy of brodalumab in subjects with rheumatoid arthritis and an inadequate response to methotrexate. *J Rheumatol*. **42**(6): 912-9.

Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, Cosmi L, Lunardi C, Annunziato F, Romagnani S, Cassatella MA. (2010). Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood.* **115**(2): 335-43.

Pierce SK, Morris JF, Grusby MJ, Kaumaya P, van Buskirk A, Srinivasan M, Crump B, Smolenski LA. (1988). *Immunol Rev.* **106:** 149-80.

Pirronello F, Leipe J, Schulze-Koops H, Skapenko A. Plasticity of CD4 T cells in rheumatoid arthritis (2012). Poster Sessions, European Congress of Immunology 2012.

Piper C, Pesenacker AM, Bending D, Thirugnanabalan B, Varsani H, Wedderburn LR, Nistala K. (2014). T cell expression of granulocyte-macrophage colony-stimulating factor in juvenile arthritis is contingent upon Th17 plasticity. *Arthritis Rheumatol.* **66**(7): 1955-60.

Puccetti P, Grohmann U. (2007). IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-kappaB activation. *Nat Rev Immunol.* **7:** 817-23.

Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M, Gumbleton M, Toulon A, Bodemer C, El-Baghdadi J, Whitters M, Paradis T, Brooks J, Collins M, Wolfman NM, Al-Muhsen S, Galicchio M, Abel L, Picard C, Casanova JL. (2011). Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science.* **332**(6025): 65-8.

Ramming A, Druzd D, Leipe J, Schulze-Koops H, Skapenko A. (2012). Maturation-related histone modifications in the PU.1 promoter regulate Th9-cell development. ***Blood***. **119**(20): 4665-74.

Read S, Malmström V, Powrie F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. **192**(2): 295-302.

Richter A, Löhning M, Radbruch A. (1999). Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J Exp Med*. **190**(10):1439-50.

Rivino L, Gruarin P, Häringer B, Steinfelder S, Lozza L, Steckel B, Weick A, Sugliano E, Jarrossay D, Kühl AA, Loddenkemper C, Abrignani S, Sallusto F, Lanzavecchia A, Geginat J. (2010). CCR6 is expressed on an IL-10-producing, autoreactive memory T cell population with context-dependent regulatory function. *J Exp Med.* **207**(3): 565-77.

Roeder RG. (2005). Transcriptional regulation and the role of diverse coactivators in animal cells. *FEBS Lett*. **579**(4): 909-15.

Rosen SD. (2004). Ligands for L-selectin: homing, inflammation, and beyond. *Annu Rev Immunol.* **22**: 129-56.

Rouvier E, Luciani MF, Mattéi MG, Denizot F, Golstein P. (1993). CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J Immunol.* **150**(12): 5445-56.

Ruthenburg AJ, Allis CD, Wysocka J. (2007). Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol Cell*. **25**(1): 15-30.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* **155**(3): 1151-64.

Sakaguchi S, Yamaguchi T, Nomura T, Ono M. (2008). Regulatory T cells and immune tolerance. **133**(5): 775-87.

Salgado E, Bes-Rastrollo M, de Irala J, Carmona L, Gómez-Reino JJ. (2015). High Sodium Intake Is Associated With Self-Reported Rheumatoid Arthritis: A Cross Sectional and Case Control Analysis Within the SUN Cohort. *Medicine (Baltimore)*. **94**(37): e924.

Sallusto F, Zielinski CE, Lanzavecchia A. (2012). Human Th17 subsets. *Eur J Immunol.* **42**(9): 2215-20.

Santarlasci V, Maggi L, Capone M, Frosali F, Querci V, De Palma R, Liotta F, Cosmi L, Maggi E, Romagnani S, Annunziato F. (2009). TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. *Eur J Immunol.* **39**(1): 207-15.

Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, Fazekas de St Groth B. (2006). Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. **203**(7): 1693-700.

Seko Y, Cole S, Kasprzak W, Shapiro BA, Ragheb JA. (2006). The role of cytokine mRNA stability in the pathogenesis of autoimmune disease. *Autoimmun Rev.* **5**(5): 299-305.

Shapiro L, Dinarello CA. (1995). Osmotic regulation of cytokine synthesis in vitro. *Proc Natl Acad Sci U S A*. **92**(26): 12230-4.

Shirota H, Sano K, Hirasawa N, Terui T, Ohuchi K, Hattori T, Tamura G. (2002). B cells capturing antigen conjugated with CpG oligodeoxynucleotides induce Th1 cells by elaborating IL-12. *J Immunol.* **169**(2): 787-94.

Singer A, Adoro S, Park JH. (2008). Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol.* **8**(10): 788-801.

Singh JA, Furst DE, Bharat A, Curtis JR, Kavanaugh AF, Kremer JM, Moreland LW, O'Dell J, Winthrop KL, Beukelman T, Bridges SL Jr, Chatham WW, Paulus HE, Suarez-Almazor M, Bombardier C, Dougados M, Khanna D, King CM, Leong AL, Matteson EL, Schousboe JT, Moynihan E, Kolba KS, Jain A, Volkmann ER, Agrawal H, Bae S, Mudano AS, Patkar NM, Saag KG. (2012). 2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. *Arthritis Care Res (Hoboken)*. **64**(5): 625-39.

Sundström B, Johansson I, Rantapää-Dahlqvist S. (2015). Interaction between dietary sodium and smoking increases the risk for rheumatoid arthritis: results from a nested case-control study. *Rheumatology (Oxford)*. **54**(3): 487-93.

Starr TK, Jameson SC, Hogquist KA. (2003). Positive and negative selection of T cells. *Annu Rev Immunol.* **21**: 139-76.

Starnes T, Robertson MJ, Sledge G, Kelich S, Nakshatri H, Broxmeyer HE, Hromas R. (2001). Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J Immunol.* **167**(8): 4137-40.

Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, Gerlitzki B, Hoffmann M, Ulges A, Taube C, Dehzad N, Becker M, Stassen M, Steinborn A, Lohoff M, Schild H, Schmitt E, Bopp T. (2010). *Immunity*. **33**(2): 192-202.

Storage SS, Agrawal H, Furst DE. (2010). Description of the efficacy and safety of three new biologics in the treatment of rheumatoid arthritis. *Korean J Intern Med.* **25**(1): 1-17.

Sundström B, Johansson I, Rantapää-Dahlqvist S. (2015). Interaction between dietary sodium and smoking increases the risk for rheumatoid arthritis: results from a nested case-control study. *Rheumatology (Oxford)*. **54**(3): 487-93.

Surh CD, Sprent J. (1994). T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature.* **372**(6501): 100-3.

Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. (2006). A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med.* **203**(7): 1685-91.

Tang W, Wang H, Claudio E, Tassi I, Ha HL, Saret S, Siebenlist U. (2014). The oncoprotein and transcriptional regulator Bcl-3 governs plasticity and pathogenicity of autoimmune T cells. *Immunity*. **41**(4):555-66.

Tao X, Constant S, Jorritsma P, Bottomly K. (1997). Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J Immunol.* **159**(12): 5956-63.

Teng MW, Bowman EP, McElwee JJ, Smyth MJ, Casanova JL, Cooper AM, Cua DJ. (2015). IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med*. **21**(7): 719-29.

Thomas RM, Gamper CJ, Ladle BH, Powell JD, Wells AD. (2012). De novo DNA methylation is required to restrict T helper lineage plasticity. *J Biol Chem*. **287**(27): 22900-9.

Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM. (2009). GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* **106:** 13445-50.

Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. (2009). Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol*. **10**(8): 864-71.

Unanue ER. (1984). Antigen-presenting function of the macrophage. *Annu Rev Immunol.* **2**: 395-428.

Underhill DM, Bassetti M, Rudensky A, Aderem A. (1999). Dynamic interactions of macrophages with T cells during antigen presentation. *J Exp Med.* **190**(12): 1909-14.

Valzasina B, Guiducci C, Dislich H, Killeen N, Weinberg AD, Colombo MP. (2005). Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood*. **105**(7): 2845-51.

Veldhoen M, Hocking RJ, Flavell RA, Stockinger B. (2006). Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nat Immunol.* **7**(11): 1151-6.

Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, Stockinger B. (2008). Transforming growth factor-beta 'reprograms' the

differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*. **9**(12): 1341-6.

Wang X, Fujita M, Prado R, Tousson A, Hsu HC, Schottelius A, Kelly DR, Yang PA, Wu Q, Chen J, Xu H, Elmets CA, Mountz JD, Edwards CK 3rd. (2010). Visualizing CD4 T-cell migration into inflamed skin and its inhibition by CCR4/CCR10 blockades using in vivo imaging model. *Br J Dermatol*. **162**(3): 487-96.

Wei L, Laurence A, Elias KM, O'Shea JJ. (2007). IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem.* **282**(48): 34605-10.

Wei G, Wei L, Zhu J, Zang C, Hu-Li J, Yao Z, Cui K, Kanno Y, Roh TY, Watford WT, Schones DE, Peng W, Sun HW, Paul WE, O'Shea JJ, Zhao K. (2009). Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity*. **30**(1): 155-67.

Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F, Lecron JC, Kastelein RA, Cua DJ, McClanahan TK, Bowman EP, de Waal Malefyt R. (2007). Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol.* **8**(9): 950-7.

Witowski J, Pawlaczyk K, Breborowicz A, Scheuren A, Kuzlan-Pawlaczyk M, Wisniewska J, Polubinska A, Friess H, Gahl GM, Frei U, Jörres A. (2000). IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. *J Immunol.* **165**(10): 5814-21.

Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, Regev A, Kuchroo VK. (2013). Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature*. **496**(7446): 513-7.

Xie MH, Aggarwal S, Ho WH, Foster J, Zhang Z, Stinson J, Wood WI, Goddard AD, Gurney AL. (2000). Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. *J Biol Chem.* **275**(40): 31335-9.

Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, Kuchroo VK, Hafler DA. (2008). IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature.* **454**(7202): 350-2.

Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang YH, Schluns KS, Broaddus RR, Zhu Z, Dong C. (2008). Regulation of inflammatory responses by IL-17F. *J Exp Med.* **205**(5): 1063-75.

Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, Dong C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem.* **282**(13): 9358-63.

Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, Cohen JI, Spriggs MK. (1995). Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity.* **3**(6): 811-21.

Yi H, Zhen Y, Jiang L, Zheng J, Zhao Y. (2006). The phenotypic characterization of naturally occurring regulatory CD4+CD25+ T cells. *Cell Mol Immunol*. **3**(3): 189-95.

Zhang F, Meng G, Strober W. (2008). Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat Immunol*. **9**(11):1297-306.

Zhao X, Su J, Wang F, Liu D, Ding J, Yang Y, Conaway JW, Conaway RC, Cao L, Wu D et al. (2013). Crosstalk between NSL histone acetyltransferase and MLL/SET complexes: NSL complex functions in promoting histone H3K4 di-methylation activity by MLL/SET complexes. *PLoS Genet.* **9:** e1003940.

Zheng Y, Rudensky AY. (2007). Foxp3 in control of the regulatory T cell lineage. *Nat Immunol.* **8**(5): 457-62.

Zhou Q, Desta T, Fenton M, Graves DT, Amar S. (2005). Cytokine profiling of macrophages exposed to Porphyromonas gingivalis, its lipopolysaccharide, or its FimA protein. *Infect Immun.* **73**(2): 935-43.

Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. **8**(9): 967-74.

Zhou Q, Haupt S, Prots I, Thummler K, Kremmer E, Lipsky PE, Schulze-Koops H, Skapenko A. (2013). miR-142-3p is involved in CD25+ CD4 T cell proliferation by targeting the expression of glycoprotein A repetitions predominant. *J Immunol.* **190**(12): 6579-88.

Zhou XY, Yashiro-Ohtani Y, Nakahira M, Park WR, Abe R, Hamaoka T, Naramura M, Gu H, Fujiwara H. (2002). *J Immunol.* **168**(8): 3847-54.

Zhu J, Yamane H, Paul WE. (2010). Differentiation of effector CD4 T cell populations. *Annu Rev Immunol.* **28**: 445-89.

Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, Monticelli S, Lanzavecchia A, Sallusto F. (2012). Pathogen-induced human TH17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. *Nature.* **484**(7395): 514-8.

Zrioual S, Toh ML, Tournadre A, Zhou Y, Cazalis MA, Pachot A, Miossec V, Miossec P. (2008). IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR+ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. *J Immunol*. **180**(1): 655-63.

ACKNOWLEDGEMENTS

I would like to thank PD Dr. Alla Skapenko, Dr. Jan Leipe and Prof. Dr. Hendrik Schulze-Koops for introducing me to the fascinating subject of immunology. I would also like to thank them for giving me the opportunity to conduct my thesis work in the research laboratory of the Division of Rheumatology and Clinical Immunology of the Klinikum der Universität München, and for giving me the wonderful opportunity to participate to, and present my work at national and international scientific meetings.

I would particularly like to thank PD Dr. Alla Skapenko for the time and effort spent in guiding me, on a daily basis through the complexities of the subject of my research project, for always being available to give detailed scientific and technical explanations, for indicating the path throughout the project, and for reviewing my PhD thesis.

I thank my co-supervisor Dr. Jan Leipe for helping in planning and initiating my project, for teaching me many research techniques and giving me, through his experience as a researcher and medical doctor, insights on the very interesting clinical aspects of the subject of my research. I would also like to thank him for the time dedicated to reviewing my PhD thesis.

I would like to thank Prof. Dr. Stefan Endres for allowing me to participate to the Research Training Group DFG Graduiertenkolleg 1202.

I thank Dr. Matthias Schiemann and Prof. Dirk Busch for the initial cooperation on using the cell sorters in his facility and to Lynette Henkel for her technical assistance.

I would like to thank all the colleagues and the dedicated and wonderful people I had the pleasure to work with over the years, PhD students and postdocs, technical assistants, medical doctors and students.

I am extremely grateful to my parents and my sister for the unconditional love and support they offered me over the years, to Matilde for the patience, encouragement and for always believing in me, and to all of my friends for the pleasant times together, the laughs and the support.