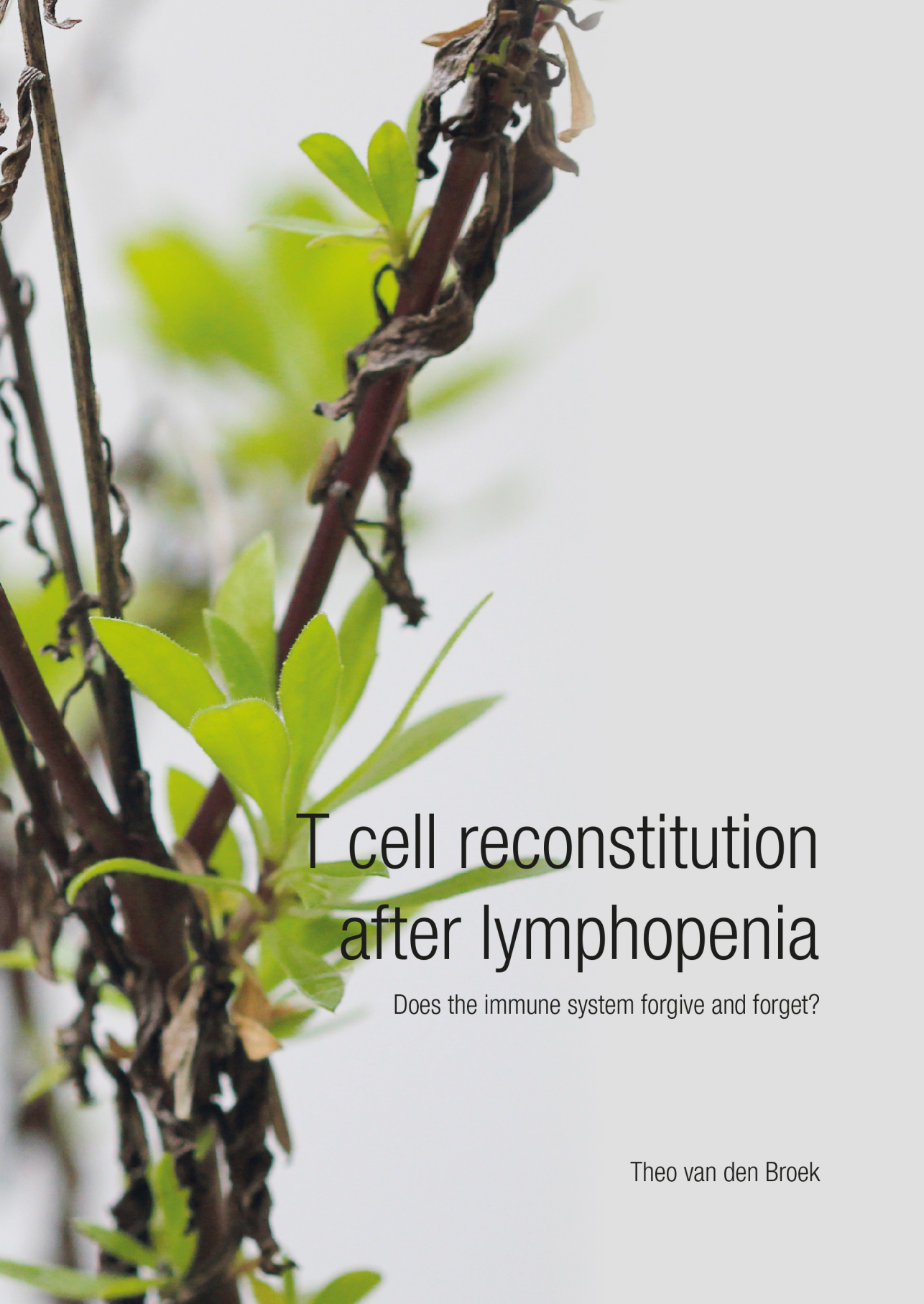

T cell reconstitution after lymphopenia

Does the immune system forgive and forget?

Theo van den Broek

T cell reconstitution after lymphopenia
Does the immune system forgive and forget?

PhD thesis, University of Utrecht, the Netherlands, with a summary in Dutch

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T cell reconstitution after lymphopenia

Does the immune system forget and forget?

Blijft T cel reconstitutie na lymfopenie zonder consequenties?

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
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Theodorus van den Broek

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Promotor: Prof.dr. A.B.J. Prakken

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PART TWO

ADDENDA

1

General introduction

PART ONE

The adaptive immune system is a dynamic organ that is continuously changing and adapting to different triggers from within and outside the human body. There are two main broad classes of adaptive immunity; antibody responses and cell mediated immune responses that are also carried out by two different lymphocytes (B cells and T cells respectively). While B cells are generated in the bone marrow, the thymus is responsible for the generation of new naïve T cells. As early as 12 weeks of gestation the neonatal thymus starts producing T cells, resulting in a diverse repertoire of naïve T cells at birth. [1-5] The role of the thymus in the establishment of a normal immune system has been profoundly studied in mice. These studies have greatly contributed to the understanding of its role in health and disease in humans. [6] Even though, the dynamics of thymopoiesis and the thymic contribution towards the immune system greatly differs between mice and men. [7, 8] In mice thymus production starts around birth and production of naïve T cells is largely sustained during aging, while in humans the thymus declines in size and function, termed thymic involution, and the naïve T cell pool is primarily maintained by peripheral homeostatic proliferation in adults. [7, 9-12] Human T cell immune homeostasis is therefore dependent upon the contributions of two primary pathways: generation of new T cells from progenitors via thymopoiesis, and peripheral expansion of residual lymphocytes (homeostatic proliferation [HP]). [8] The process of T cell reconstitution involves a dynamic balance between the two pathways. Peripheral expansion pathways are immediate, while the impact of the thymus is much slower. [13] The cumulative contribution from each pathway may vary depending upon host age, residual T cell subsets, homeostatic cytokine levels and endogenous antigenic stimulation. [13-15] The sequelae of the two pathways are also quite disparate, resulting in either a diverse T cell repertoire through active thymopoiesis or a skewed, oligoclonal, peripherally-derived population. [16-18]

There are two main types of homeostatic proliferation: a slow, non-exogenous antigen driven process which occurs in response to a "sensing of empty space" and a fast, antigen-specific process, undergone by a smaller subset of T cells that is likely driven by presentation of antigens from commensal bacteria and is independent of IL-7. [19-21] The maintenance of the T cell pool via slow HP depends on receiving homeostatic signals, predominantly IL-7 and self-peptide-Major Histocompatibility Complex (MHC complexes). In the case of T cell lymphopenia the same factors that are crucial for supporting naive T cell homeostasis in a 'non-lymphopenic' environment are also vital for the *lymphopenia- induced (homeostatic) proliferation* (LIP) response. [21-24] However, greater access to these factors and/or enhanced intensity of their signals is thought to drive T cell expansion and differentiation in a lymphopenic environment, promoting naive T cell proliferation and gradual differentiation towards a memory-like state. [21-24] Thus LIP preferentially expands T cells with a greater specificity and a stronger avidity

for self, potentially resulting in excessive autoreactivity. [19, 20, 25, 26]
The role of thymopoiesis and (lymphopenia-induced) homeostatic proliferation has been studied in several human and murine models of T cell lymphopenia, but in this thesis we will focus on the dynamics of T cell reconstitution in the context of stem cell transplantation in a murine model and in children with an autoimmune disease, and after neonatal thymectomy in children and adolescents.

Role of the thymus

T-cell development occurs in the thymus, where thymocytes undergo phenotypic changes and acquire their repertoire for the antigen-specific T cell receptor (TCR). To ensure that the specificity of the new TCR is adequate, T cells need to undergo two selection processes. First, involves recognition of self-MHC; those with no interaction are destroyed (positive selection). Second, T cells with a high affinity interaction towards 'self-antigens' are eliminated (negative selection), to reduce autoreactivity. In the end, resulting in a naïve T cell pool with a broad TCR repertoire able to recognize antigens presented in self-MHC, but with low reactivity towards self-antigens. Another thymic-derived subpopulation of T cells are regulatory T cells (natural Treg, nTreg). These cells are crucial for immune homeostasis and self-tolerance and are suggested to be selected in thymus for having an intermediate affinity for self-peptide. [27]
In children, the T cell compartment grows continuously with age from birth to adulthood. Cell numbers then remain approximately stable throughout adult life. During the first 20 years of life, the thymus is known to involute, and its output is supplemented by division from within the existing peripheral naive T cell pool. [12, 28-32] New naïve T cells with a broad TCR repertoire can only be generated by thymic output, while peripheral proliferation results in the expansion of the existing naïve T cell pool. In the case of regulatory T cells, thymic-derived Treg are likely maintained by peripheral proliferation, but it has become clear that conventional non-Treg T cells can differentiate in the periphery to become Treg cells that are known as induced Treg cells (iTreg). [33] The naïve and regulatory T cell subsets are further introduced in the following sections.

Naïve (CD4) T cells

Naïve T cells were considered a relative homogenous population of T cells not having encountered their respective antigen, only differing in their TCR reactivity. With the characterization of CD31 expression on naïve T cells, a division in naïve T cells that are enriched in recent thymic emigrants (CD31+) and those that have peripherally expanded via antigen recognition (CD31-) has been made.[35] This concept of naïve T cell division is supported by the immunological changes seen during aging. With aging, the CD31+ naïve T cell subset decreases with thymic output, while the CD31- naïve T cell subset increases and maintains the total naïve T cell compartment over time. [16, 34, 35] The idea that naïve

T cells have not yet engaged in TCR engagement is therefore no longer appropriate. Specific cytokine production as seen in other T helper subsets after antigen recognition and differentiation are absent or low expressed, with certain exceptions as interleukin-2 (IL-2) and TNFalpha. [36, 37] Recently, IL-8 has been described as a signature cytokine of naïve T cells in neonates. [36] These neonatal naïve T cells express markers characteristic for CD31+ naive CD4+ T cells. IL-8 is the most documented effector cytokine of naïve neonatal CD4+ T cells and is suspected to be involved in protective mechanisms against infection in the neonatal phase. This subset however loses the expression of IL-8 during aging [36], suggesting that even further differentiation within this CD31+ CD4+ naïve T cell is existing. Functional changes within the naïve T cell compartment may have major implications for vaccination and immune intervention strategies, especially in the very young and elderly. Hence, further understanding of the naïve T cell compartment and its different functional aspects and subsets is necessary for specific intervention and monitoring.

Regulatory T cells

Next to the production of naïve T cells, a distinct type of T cell, regulatory T cell (Treg) is generated in the thymus and play an essential role in immune homeostasis, as disruption in their development or function results in autoimmunity and inflammatory diseases. [38] These Treg cells express FOXP3, a forkhead transcription factor, that is considered a lineage specific transcription factor of Treg. [39] However, FOXP3 expression alone is not sufficient for reliably delineating functional Treg cells. It has been shown that thymic-derived Treg (nTreg) have specific demethylated DNA sites that are of importance for Treg function and stability of the Treg lineage stability. [40, 41] However, current tools to measure Treg specific DNA methylation regions (TSDR) require a rather large amount of cells that makes it difficult to assess Treg stability in neonates and children of whom only small amounts of material can be gathered. [42, 43] Thus, adequate characterization of Treg cells is not possible in young children or patients with low T(reg) cell amounts. However, this is needed to further understand the development and maintenance of Treg throughout life. Next to, thymic-derived Treg (nTreg) there is evidence that part of the circulating Treg cells are developed from non-Treg cells. [44, 45] In several murine models, these induced Treg (iTreg) were seen after adoptive transfer of non-Treg cells into lymphopenic mice. [46-49] In subsequent homeostatic proliferation some of the transferred non-Treg cells acquire phenotypical and functional characteristics of nTreg cells. In human it is not yet possible to distinguish nTreg and iTreg from each other to provide further insight into Treg dynamics. [50-52] So far, human Treg can be divided in three different subsets depending on the expression of CD45RA (or CD45RO) and FOXP3. [53] The Treg subsets with functional suppressive capacity (FOXP3+CD45RA+, resting Treg and FOXP3^{high}CD45RA- active Treg) and a non-suppressive population (FOXP3^{low}CD45RA-, non-Treg). The active Treg population is proposed to originate from the resting Treg population. Although they may also be induced

from non-Treg cells. [54] One of the functions attributed towards thymic-derived Treg (nTreg) is to mediate auto-reactivity in the periphery (peripheral tolerance), while iTreg have been suggested to be important in tolerance to non-pathogenic foreign antigens (such as intestinal microbiota).[55, 56] Studies on Treg dynamics are mostly focused on the adult immune system that primarily depends on peripheral proliferation. The role of the thymus in the dynamics between these Treg subsets is therefore underrepresented and is important in understanding pediatric immune homeostasis and of interest in the aging immune system that shows increased auto-reactivity.

T cell reconstitution dynamics in the lymphopenic setting

T-cell reconstitution during lymphopenia depends on two sources: (1) thymopoiesis which results in the generation of new naïve T cells; and (2) spontaneous expansion of the existing peripheral T-cell pool. The relative contribution of each pathway to replenishment of the peripheral T-cell pool varies with age, thymic function, availability of homeostatic cytokines and antigenic stimulation. Moreover, the balance between these two sources of T cells during immune reconstitution may influence T cell reactivity, clinical outcome and future immunological responses. The reconstitution dynamics in the autologous stem cell transplantation setting and after neonatal thymectomy are now further introduced and schematically summarized in figure 1.

Hematopoietic stem cell/bone marrow transplantation

Hematopoietic stem cell transplantation (HSCT) is the process of intravenous infusion of hematopoietic stem and progenitor cells to restore normal hematopoiesis after immune depletion to treat for malignant and non-malignant (hematologic disorders, congenital immunodeficiencies, inborn errors of metabolism, and autoimmunity) disease. [57] After stem cell infusion and grafting, part of the induced progenitor cells are believed to home to the thymus and undergo a selection process. This process ensures that new T cells can respond to T-cell receptor triggering, but don't have excessive auto-reactivity, termed central tolerance. [58-60] Hematopoietic stem cells obtained from the patient him- or herself are referred to as autologous. Hematopoietic stem cells obtained from an identical twin are referred to as syngeneic obtained, and hematopoietic stem cells from someone other than the patient or an identical twin are referred to as allogeneic. The goal of the treatment is to delete the faulty immune system and then repopulate the immune system from the infused new stem cells, which results in new immune cells that don't display the previous faulty characteristics.

The contribution of thymopoiesis and peripheral proliferation has been studied after T cell depletion of the recipient, as performed prior to stem cell transplantation. [59-61] In the human allogeneic HSCT setting, the donor (thymic-derived) and the host (peripheral-induced) immune system can be distinguished from each other by detecting HLA-mismatches.

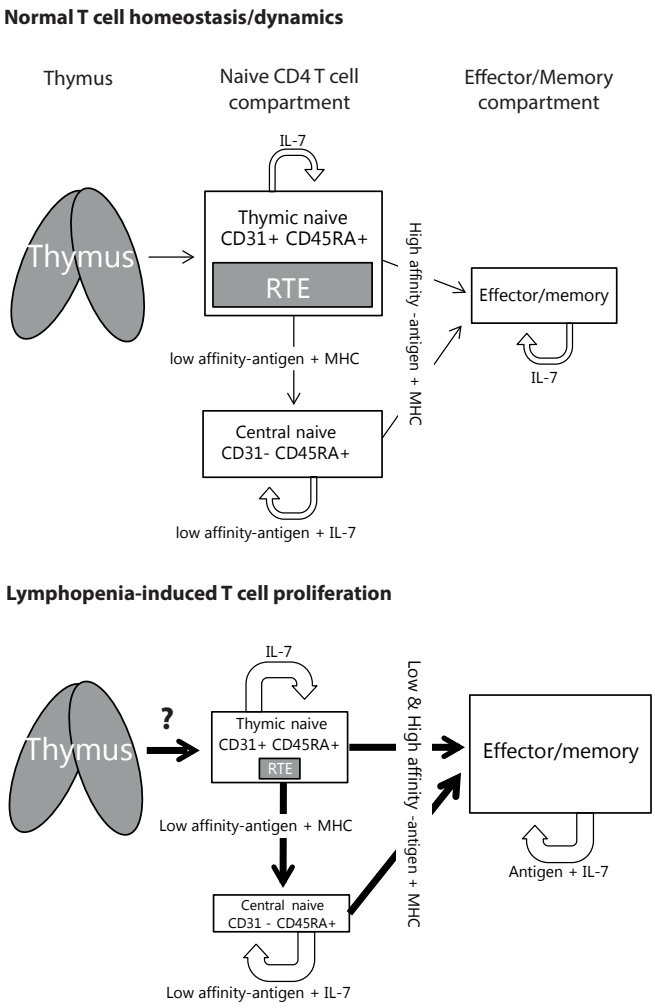

Figure 1. T cell homeostasis and dynamics in normal and lymphopenic circumstances of young individuals. Thymopoiesis results in the generation of recent thymic emigrants (RTE) that are incorporated in the thymic naïve T cell compartment. This naïve T cell compartment can peripherally expand (partly IL-7 mediated), skew towards a central naïve phenotype after low-affinity (self-)antigen recognition or towards an effector/memory phenotype after high-affinity (foreign) antigen recognition. Peripheral expansion of each compartment is present but of a lesser degree in healthy children in comparison to adults who are more dependent on homeostatic proliferation than thymopoiesis.
Some have suggested that in lymphopenia, thymopoiesis may be increased, but so far there is no evidence for such a homeostatic response of the thymus. Under lymphopenic circumstances, both the cell numbers of the naïve T cell compartment and especially the RTE are decreased with increased skewing towards effector/memory phenotype. The threshold for T cell receptor mediated skewing and proliferation is lowered in lymphopenic conditions. This results in the additional recognition of low affinity antigens, such as self-antigens, potentially leading to increased auto-reactivity. In addition, peripheral proliferation of the effector/memory compartment is of a larger magnitude than that of the naïve T cell compartment further enhancing T cell receptor oligoclonality.

The ratio of donor and host (chimerism) is essential to detect residual host hematopoiesis which could be responsible for disease relapse. This makes it possible to track reconstitution dynamics following T cell lymphopenia. However, the HLA-mismatches between host and donor cells in the allogeneic setting also causes changes in their reconstitution dynamics. As graft (donor) cells can suppress the repopulation of host cells ('graft versus host', GvH). This can be beneficial as the new stem cells (donor) can suppress any residual faulty immune cells (host). However, when in excess it could also attack the body and result in a potentially fatal graft versus host disease (GvHD). [62, 63] In the human autologous setting it is not possible to track the infused ('donor') T cell reconstitution, as they are from the same person and therefore identical to 'host' T cells. As both host and donor are alike, suppression of the disease related 'host' cells by new thymic-derived ('donor') T cells is likely diminished in comparison to the allogeneic setting. Lymphocytes that survive the lymphocyte depletion prior to stem cell transplantation can than potentially reconstitute part of the 'new' immune system. This can be one of the reasons why after several years disease relapses occur. [64, 65] In addition to the idea that stem cell transplantation just sets back the immunological clock for several years in an individual that has proven to be susceptible for this disease. How the transplants are exactly rewiring a faulty immune system is still unknown. For example, it is not clear which cells must be destroyed prior to transplantation, nor is it known which ones keep disease under control afterward. [66] This is especially the case for the autologous setting when the reconstitution dynamics of the 'host' and 'donor' T cell compartment are unknown and how this results in resolution of disease.

T cell reconstitution dynamics after neonatal thymectomy

The role of the thymus in immune homeostasis has been extensively studied in thymectomized mice [6], but greatly differs between human and mice. [7, 8] The thymus is essential for the initial establishment of the peripheral T cell pool. The human thymus gradually atrophies after the age of approximately one year at a rate of 1-3% per year. [67] Regardless of involution, the thymus continues to serve as the site of T cell maturation and production throughout adulthood. [68] In humans, removal of the thymus occurs in both children and adults for a select indications. Therapeutic removal of the thymus in adult patients with myasthenia gravis does not result in a significantly altered composition of the T cell population, probably due to the prominent role of peripheral T cell proliferation. [69-71] In infants, cardiac surgery necessitating thymic removal, to gain an unrestricted view of the operation site, does however result in an altered T cell dynamics. [72-75] In these infants, reduced T cell numbers, specifically naïve T cells, and other T cell abnormalities associated with premature immune aging, such as a delayed response to vaccination, an oligoclonal T cell repertoire, and increased markers of inflammation have been reported. [76-82] In adults, several attempts have been made to regenerate thymic tissue and hereby rejuvenate the aged immune system. Even though some progress has been made in restoring some thymic

function, full restoration of thymic function is not yet possible. [83, 84] Recently, it was shown that after neonatal thymectomy regeneration of thymic tissue is present with increased recent thymic emigrants (RTE). [82] It is for now unknown if this thymic regeneration can actually restore the naïve T cell compartment and rejuvenate the prematurely aged immune system, but is of great importance in understanding thymic function in health, disease and in immune aging.

Induction of autoreactivity during peripheral T cell reconstitution

With thymopoiesis new T cells with a broad repertoire are generated, while after peripheral proliferation an oligoclonal skewed T cell reactivity is induced. [16-18] Next to certain homeostatic cytokines, such as IL-7, antigen recognition is required for the induction peripheral proliferation. [10, 11, 85] These antigens can be derived from foreign antigens or from self-antigens, creating the possibility of developing T cell autoreactivity. [86-88] An association between lymphopenia and autoimmunity is evident and could be due to the peripheral T cell expansion via self-antigen recognition. [89-91] In the case of therapeutic lymphocyte depletion for multiple sclerosis or prior to stem cell transplantation, development of a secondary autoimmune disease is documented and suggested to be related to lymphopenia-induced proliferation. [86, 87, 92-94] However, these associations have mostly been made in settings of a prior immune disturbance, such as auto-immunity and primary immune deficiencies. It is believed that the development of (primary) autoimmunity is the result of a combination of factors such as genetic predisposition or mutations and failure of various regulatory features. [95-97] It could therefore be that the threshold for the development of a (secondary) autoimmunity as result of lymphopenia-induced proliferation is lowered in patients with pre-existing autoimmunity in comparison to healthy individuals. Thus the induction of autoreactivity and autoimmunity via lymphopenia-induced proliferation is uncertain in the context of an immunocompetent individual.

PART TWO

Immunological biomarkers in rheumatoid and juvenile arthritis

In rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA), an autoimmune disease predominantly focused on the joints, the initial trigger(s) that resulted in autoimmunity is unknown and patients are treated with immune modulating, suppressive drugs and as a last resort treatment via stem cell transplantation. [98-100] A wide variety of markers related to disease development, progress, and severity, and for monitoring therapy response have been documented and can be divided into several categories, such clinical markers, imaging markers, and a large group of biochemical markers. With the introduction of 'biologicals' the range of drugs that can be used in rheumatoid arthritis has expanded, but creates a new

need to identify markers that help identify not only response to treatment but also susceptibility to it. In addition, RA and JIA are both heterogeneous in presentation, prognosis and treatment among patients, the use of personalized medicine guided via certain (bio)markers might result in a larger group of clinical responders. [101]

Juvenile Idiopathic Arthritis (JIA), being arthritis for more than 6 weeks in a child before the 16th birthday, is not a single homogeneous disease, but instead consists of different subtypes with possible distinct immune pathogenesis. The current classification for JIA is based on the International League of Associations for Rheumatology (ILAR) classification. [102] This classification separates the disease entity JIA into 7 disease categories based on features (e.g. number of joints) present in the first 6 months of disease. [102] Part of the current classification separates the disease entity by the number of joints involved, which might not be an adequate criterion for identifying homogeneous disease entities but rather reflects disease activity. [102-104] The combination of the determinants, young age at disease onset combined with ANA-positive antibodies seems to better classify a homogeneous subset of JIA patients than by their number of involved joints. [105, 106] It is uncertain why this group of patients present as a homogenous group with characteristics such as a female predominance, increased risk of developing uveitis, and asymmetric arthritis. Underlying immunological differences might be involved and are of interest as they could serve as a potential target for intervention or as biomarkers for prediction and classification of disease and treatment response. Possibly resulting in a decrease of disease duration and subsequent joint destruction.

Outline of this thesis

This thesis primarily describes the dynamics of T cell reconstitution as a result of T cell lymphopenia. Both thymopoiesis and peripheral T-cell expansion contribute to T cell homeostasis. However, several factors, such as age of the host, the amount of T cells left, and triggers needed for expansion have a different impact and result on T cell reconstitution. We initially focus on the effect of neonatal thymectomy on the homeostasis of T cell populations by peripheral proliferation and thymopoiesis. *Chapter two* addresses the plasticity of the human Treg population after neonatal thymectomy, with specific focus on two distinct Treg subpopulations. In *chapter three* we address the phenotypical and functional heterogeneity in the naïve T cell compartment in the course of years after neonatal thymectomy. *Chapter four* describes an optimized method to measure Treg specific demethylation regions (TSDR) that provides new possibilities for Treg analysis in scarce human samples. *Chapter five* investigates the role of peripheral proliferation in the induction of autoreactivity in immunocompetent children lacking a thymus.

We than continue to study the dynamics of T cell reconstitution of both the host and donor compartment in the context of bone marrow transplantation post-lymphocyte depletion. *Chapter seven*, describes the dynamics of both donor and host effector T cells after syngeneic

bone marrow transplantation in a murine model of autoimmune arthritis. *Chapter six*, focuses on regulatory T cell dynamics in the model described in chapter seven, and addresses a therapeutic approach by suppressing potentially auto-reactive host T cells early after T cell depletion through infusion Treg cells with the bone marrow transplant. In addition, we show that the broadening of the human Treg TCR repertoire is needed for clinical response after aHSCT.

In the second part of this thesis, we describe the role of immunological biomarkers in rheumatoid (RA) and juvenile arthritis. In *Chapter eight* an overview of the literature regarding biomarkers in RA is described. *Chapter nine* describes the use of soluble immunological biomarkers to identify a homogenous group of JIA patients that overlap current classification criteria.

In *Chapter ten* the findings described in this thesis as well as future directions are discussed.

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PART ONE

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2

Differential homeostatic dynamics of human regulatory T-cell subsets following neonatal thymectomy

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FOXP3-expressing $CD4^+$ regulatory T (Treg) cells are important in the maintenance of self-tolerance and immune homeostasis. There are 2 possible origins for Treg cells: (1) thymus-derived natural Treg (nTreg) cells and (2) peripherally induced Treg (iTreg) cells.[1] Although both Treg cell populations express similar phenotypic proteins, it has been proposed that they exert different functions in maintaining immune homeostasis. While nTreg cells have been shown to be essential in self-tolerance, iTreg cells may be important in tolerance to nonpathogenic foreign antigens.[2] Disturbing the production of either Treg cell population may affect immune regulation later in life. Recently, it has been shown, for example, that alterations at neonatal age in thymic Treg cell maturation affects clinical outcome.[3] It was demonstrated that in atopic children, thymic Treg cell function is significantly delayed early on in life. However, further data on human Treg cell development early in life are scarce.

The functional Treg cell population contains 2 distinct populations, a naive $CD45RA^+RO^-$ FOXP3low fraction and an activated/memory $CD45RA^+RO^-$FOXP3high fraction, both equally capable of suppressive activity.[4] Although both subpopulations are true Treg cells, they have distinct differentiation dynamics. We hypothesized that Treg cell population dynamics would be affected in patients who undergo neonatal thymectomy during cardiac surgery. Previously, we showed the effect of neonatal thymectomy on long-term restoration of the naive T-cell compartment.[5] In the present study, we evaluated the dynamics of distinct Treg cell subpopulations in the first 3 years following neonatal thymectomy.

Twenty-six children with a median age of 11.4 months (range, 2.5-34.7 months) who were previously thymectomized during the correction of a cardiac defect were included (Table E1). The study was approved by the medical ethical committee of the University Medical Center Utrecht (METC 05-041 and 06-149), and written informed consent was obtained. Thymectomy was performed within the first month of life (10.0 - 9.0 days) in all participants. At the time of blood sampling, all children showed no sign of infection or immune dysregulation. For full information on the study population and flow cytometry staining protocols, see this article's Methods section in the supplemental information.

First, we determined the impact of thymectomy on the total, peripheral CD4+ T-cell population. Following thymectomy, absolute CD4 counts dropped significantly compared with those in healthy infants (Figure 1A and Figure E1A). Similarly, total $FOXP3^+CD4^+$ T-cell numbers were significantly lower in thymectomized patients than in healthy age-matched controls (Figure 1B, and Figure E1B). At a young age, $CD31^+$(PECAM-1) T cells represent recent thymic emigrants.[6] Compatible with a loss of thymic production of Treg cells, thymectomized patients showed a significantly lower percentage of $CD31^+FOXP3^+$ T cells than did age-matched controls (Figure 1C). Taken together, neonatal thymectomy results in a loss of thymus derived Treg cells and a reduced number of circulating Treg cells.

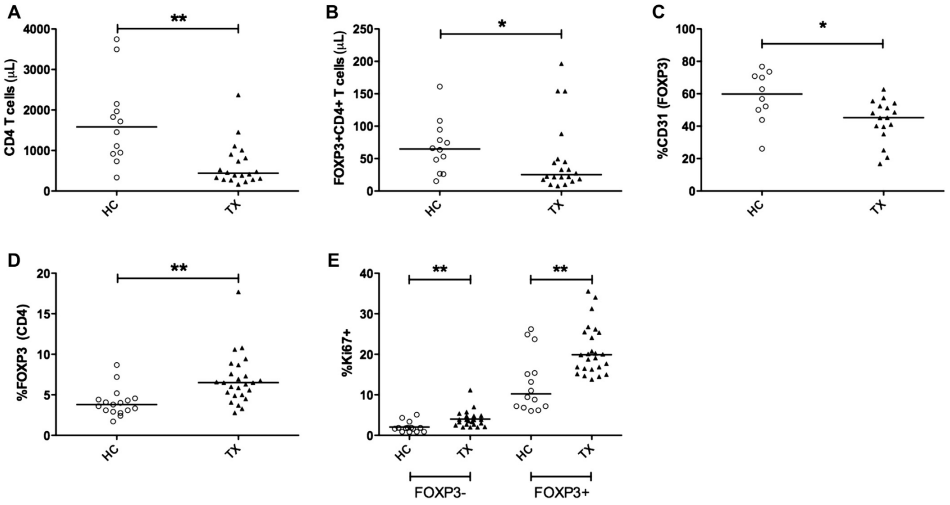

Figure 1. CD4⁺ and FOXP3⁺ T-cell dynamics and expression of CD31 and Ki67 after thymectomy. A, CD4+ T-cell count. B, FOXP3⁺CD4⁺ Treg-cell count. C, Percentage of CD31⁺ (FOXP3⁺) Treg cells. D, Percentage of FOXP3⁺ (CD4⁺) cells. E, Percentage of FOXP3⁻ and FOXP3⁺ T cells in cell cycle (Ki67⁺) in thymectomized subjects (TX) (O) and age-matched controls (HC) (B). Horizontal line represents median value per group. *P < .05 and **P < .001.

Interestingly, after thymectomy, the percentage of FOXP3⁺CD4⁺ T cells was increased compared with that in controls (Figure 1D, and Figure E1C). Therefore, we investigated whether there was an increase in peripheral proliferation to compensate for the loss of thymic output. Compared with the FOXP3⁻CD4⁺ population, the FOXP3⁺CD4⁺ Treg cell population had a higher proportion of proliferation marker Ki67⁺ cells, with a significant increase following thymectomy (Figure 1E). Thus, these data suggest that there is a compensatory, selective expansion of Treg cells, leading to an increased percentage of Foxp3⁺CD4⁺ T cells.

Next, we studied the dynamics of 2 distinct Treg cell subpopulations; gating of the FOXP3⁺ fractions is illustrated in Figs E2 and E3. The naive subpopulation CD45RO⁻FOXP3low (fraction I) and the memory population with the highest FOXP3 expression CD45RO⁺FOXP3high (fraction II) have been shown to be "true" Treg cells, whereas the memory population with low FOXP3 expression (fraction III) is nonsuppressive.[4] We observed that the percentage of FOXP3 cells expressing the naive CD45 isoform (fraction I) remained stable, whereas both memory fractions increased significantly in thymectomized patients (Figure 2A).

The relative increase in the heterogeneous and non-suppressive CD45RO$^+$FOXP3low population (fraction III) may be a reflection of activation-induced transient upregulation of FOXP3, as has been shown in vitro.[7] Absolute numbers of both "true" Treg cell fractions decreased marginally, though not significantly (Figure 2B). While numbers of CD45RO$^-$FOXP3low Treg cells (fraction I) were within the range of healthy controls, the majority of the patients had low numbers (Figure 2B). Thus, neonatal thymectomy affects the composition of the Treg cell population.

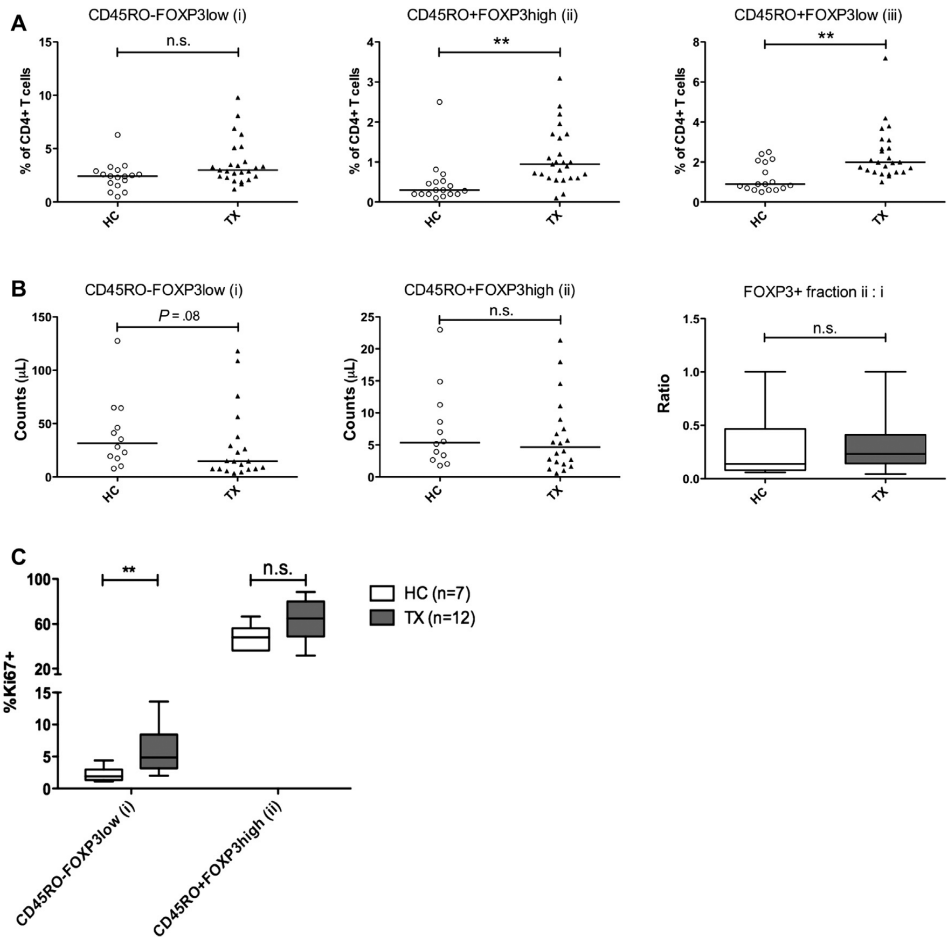

Figure 2. FOXP3$^+$ subpopulation dynamics after thymectomy. **A**, Percentage of the 3 FOXP3$^+$ subpopulations within CD4 T-cell population. **B**, Cell count of the 2 FOXP3$^+$ Treg-cell populations in healthy controls (HC) (O) and thymectomized patients (TX) (▲), and ratio of fraction ii : i. C, Expression of Ki67 per FOXP3$^+$ Treg-cell population. n.s., No significant difference. **P < .001.

When we examined the proliferation of the 2 "true" Treg cell subpopulations, a clear hierarchy was observed, with a 10-fold higher percentage of Ki67-expressing cells in CD45RO$^+$FOXP3high (fraction II) than in the naïve CD45RO$^-$FOXP3low (fraction I) Treg cell population in both thymectomized and healthy individuals. Following thymectomy, an increase in proliferation of both subpopulations was found compared to the healthy controls, reaching statistical significance in the naive Treg cell fraction (fraction I) (Figure 2C). Together, these data suggest that peripheral proliferation of both naïve and activated Treg cells compensated for the loss of thymic output, resulting in maintenance of and increase in percentages of naive Treg and activated Treg cell populations, respectively (Figure 2A). Although it is most likely that increased expansion of the activated Treg cell population is responsible for maintaining the number of activated Treg cells in these thymectomized children (Figure 2B), we cannot exclude the possibility of additional increased conversion of naive Treg cells to activated CD45RO$^+$FOXP3high Treg cells.[4] In a subgroup of patients, the number of naive CD45RO$^-$FOXP3low Treg cells was low despite increased proliferation, which appeared most prominent in the children older than 6 months (Figure E4). A shift in the balance between naive and memory Treg cells has been associated with several pathological conditions. Reduced naive Treg cells with a compensatory increase in memory Treg cells has been associated with multiple sclerosis[8] and sarcoidosis,[4] while an increase in naive Treg cells, albeit with impaired suppressive function, has been observed in active systemic lupus erythematosus.[4,9] Thus, removal of the thymus in the first month of life may affect immune regulation later in life. Overall, it is prudent to spare thymic tissue in patients requiring congenital heart surgery when technically possible.

This study demonstrates the specific homeostatic control of 2 distinct FOXP3$^+$ Treg-cell populations. Peripheral proliferation of Treg cells counteracted the effect of loss of thymopoiesis, which illustrates the relative plasticity of the human immune system. However, changes in composition of the Treg cell population do warrant further investigation of the long-term functional effects of neonatal thymectomy following cardiac surgery.

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SUPPLEMENTAL INFORMATION

METHODS

Study population and blood specimens

Twenty-six patients who all had undergone complete thymectomy at neonatal age during surgical correction for a congenital heart defect at the children's heart center, University Medical Center Utrecht, The Netherlands, were included in this study. The thymus is routinely removed during surgery involving the major vessels, such as transposition of the great arteries, hypoplastic heart syndrome, and hypoplastic aortic arch, because of its anatomical obstruction in relation to the heart. A healthy, age-matched, control group was included from both patients admitted for correction of a heart defect that did not necessitate removal of the thymus such as ventricular septum defects (n=9) and healthy children who visited the University Medical Center Utrecht to undergo elective urologic or plastic surgery (n=8). All included patients were considered immunologically healthy because they did not have a recent history of infectious disease or a hematologic or immunologic disorder. Patients with a known syndrome or genetic disorder were excluded (eg, 22q11 deletion and trisomy 21). Characteristics of the 26 included patients and healthy controls are depicted in Table E1. Because cell counts were not available for all samples, absolute numbers of cell populations are not shown for all study subjects.

Cell preparation and flow cytometry

PBMCs were isolated from heparinized blood samples by using the Ficoll Isopaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden), and viably frozen and stored in liquid nitrogen until further processing. Characterization of the T-cell compartment was performed on thawed cryopreserved PBMCs that were washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FCS and 0.1% sodium azide) and blocked with normal mouse and rat serum. The cells were incubated in 50 mL FACS buffer containing the appropriately diluted antibodies against human CD3, CD4, CD45RO, and CD31. For intracellular staining of Ki-67 and FOXP3, the cells were first surface stained, followed by fixation and permeabilization according to the manufacturer's protocol. Antibodies against CD4 (clone SK3) and CD31 (WM59) were obtained from BD Biosciences (San Jose, CA), against CD45RO (UCHL1) from Caltag (Buckingham, United Kingdom), against Ki67 (MIB-1) from Immunotech (Marseilles, France), and against FOXP3 (PCH101) from eBioscience (San Diego, CA). Finally, stained mononuclear cells were washed twice in FACS buffer and run on an LSRII and analyzed by using FACSDiva software (BD Biosciences). The gates for the different populations were kept identical for each experiment containing both thymectomized patients and healthy controls.

Statistics

To analyze the quantitative differences between thymectomized patients and healthy age-matched controls, data only after thymectomy were included. Statistical significance between the 2 groups was assessed by using the Mann-Whitney U test for unpaired data and the x2 test for dichotomous data. Statistical difference is indicated as *P <.05 and **P <.001.

Table E1. Characteristics of the included patients and healthy controls

	Thymectomy	Control	p-value
No. of subject	26	17	
Age (mo)	11.4 ± 10.9	10.8 ± 12.6	0.62
Age at TX (d)	10.0 ± 9.0	-	
Female:male	9:17	6:11	0.96

TX, Thymectomy. Mo, months; d, days.

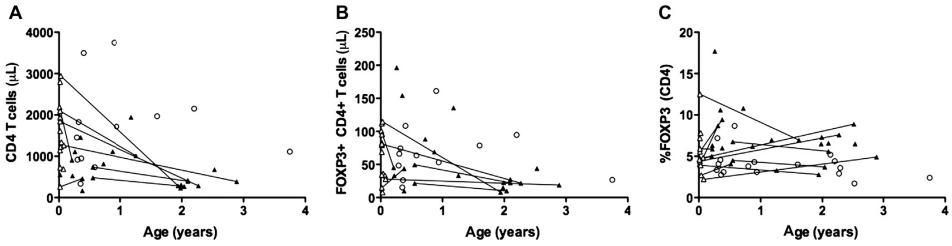

Figure E1. FOXP3 percentages and counts in the first years after neonatal thymectomy. A, Absolute $CD4^+$ T-cell counts per microliter of blood. B, $FOXP3^+CD4^+$ Treg-cell counts per microliter of blood. C, Percentage of $FOXP3^+$ cells in $CD4^+$ T-cell populations. ▲, values after thymectomy; Δ, samples taken just before thymectomy; O, healthy controls. Lines connect longitudinal samples.

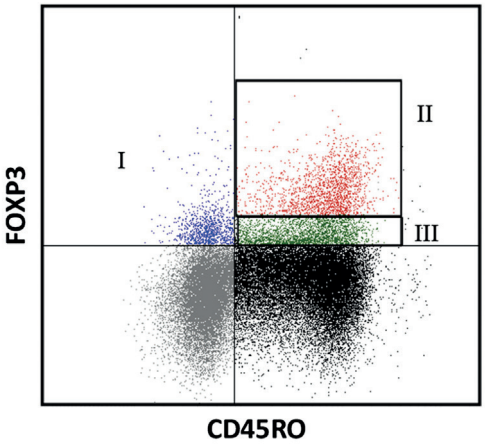

Figure E2. Gating strategy of subpopulations of FOXP3⁺ T cells.
FOXP3⁺ subpopulations after gating for CD4+ lymphocytes. Fraction I, CD45RO⁻FOXP3low; fraction II, CD45RO⁺FOXP3high; fraction III, CD45RO⁺FOXP3low.

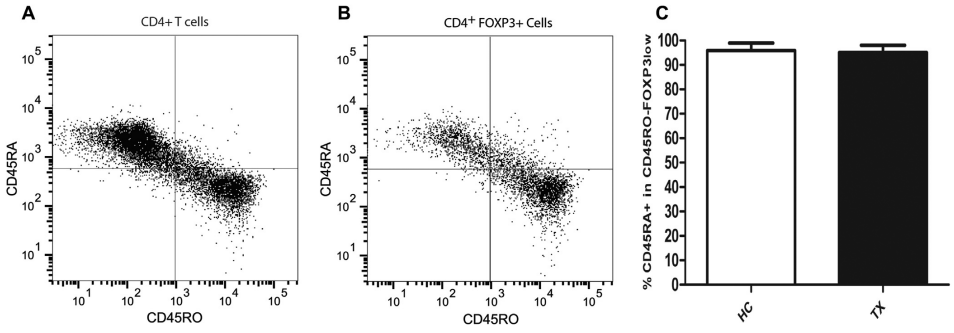

Figure E3. CD45RO⁻Foxp3⁺ T cells represent CD45RA⁺Foxp3⁺ T cells. A, Dot plot of CD45RO (memory) and CD45RA (naive) expression on CD3⁺CD4⁺ T cells. **B,** Dot plot of CD45RO and CD45RA expression on CD3⁺CD4⁺Foxp3 (Treg) cells. **C**, Expression of CD45RA⁺ in the CD45RO⁻FOXP3⁺ subpopulation. Healthy control group (HC), n=7, and thymectomized patients (TX), n=8. Data represented as mean percentage ±SD.

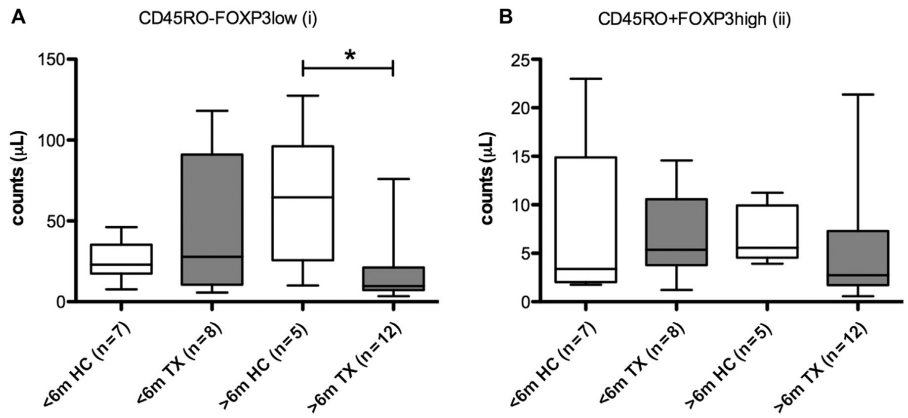

Figure E4. Subgroup analysis of naive and memory Treg-cell populations in 0- to 6-month-old and more than 6-month-old subjects. CD45RO$^-$FOXP3low naive Treg **(A)** and CD45RO$^+$FOXP3high memory Treg-cell **(B)** numbers in the subgroups less than 6 months and more than 6 months of age in healthy controls (HC) and thymectomized patients (TX). Data represented as median, 25% and 75% percentile boxes, and range.*$P < .05$.

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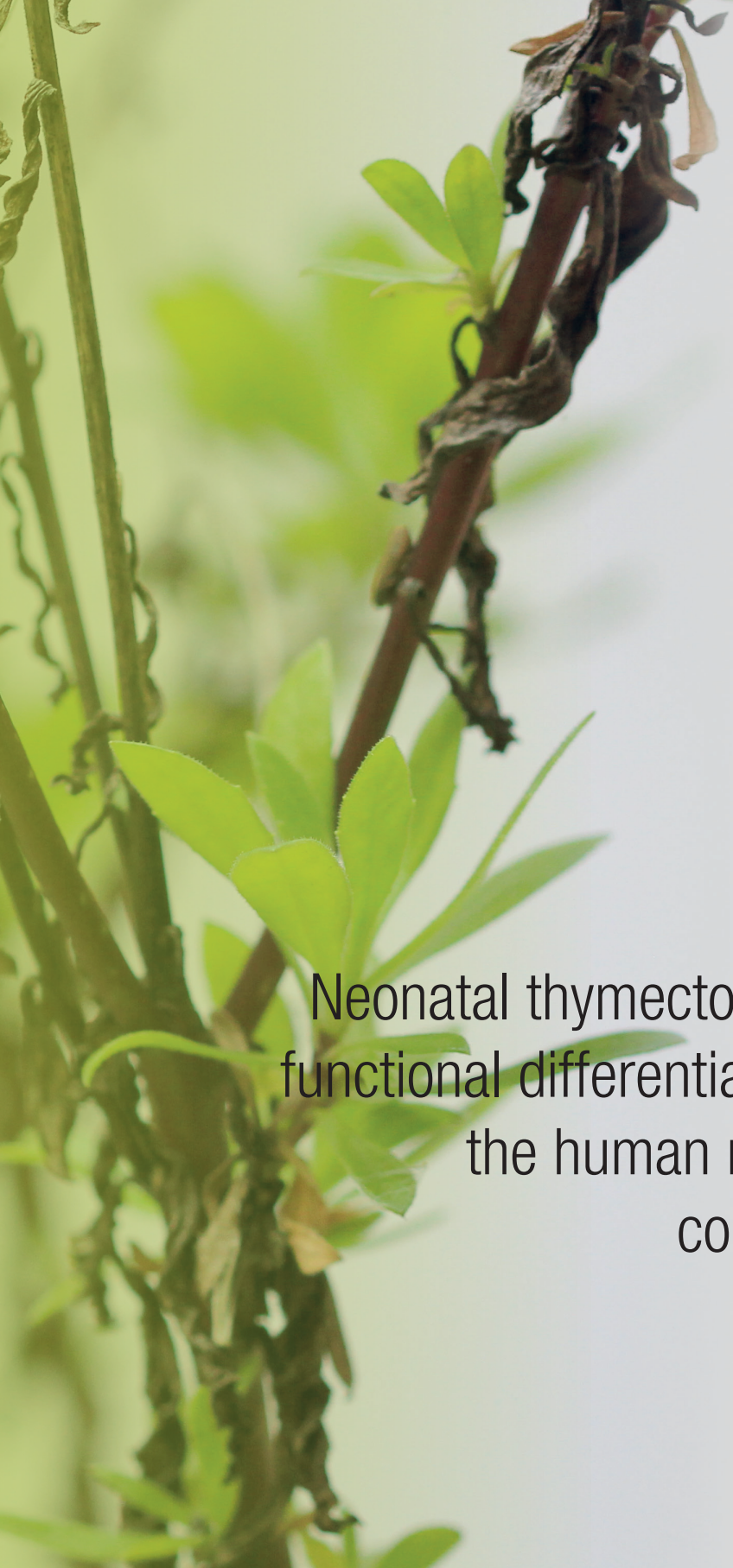


3

Neonatal thymectomy reveals functional differentiation within the human naïve T cell compartment

Submitted

SUMMARY

The generation of new naive T-cells is dependent on thymic output, but in adults the naive T-cell pool is primarily maintained by peripheral proliferation. Naive T-cells have long been regarded as relatively quiescent cells but recently it was shown that IL-8 production is a signatory effector function of naïve T-cells, at least in newborns. How this functional signature relates to naïve T-cell dynamics and aging is unknown. Using a unique cohort of children and adolescents who underwent neonatal thymectomy, we demonstrate that the naive CD4 T-cell compartment in healthy humans is functionally heterogeneous and that this functional diversity is lost after neonatal thymectomy. Thymic regeneration later in life resulted in functional restoration of the naïve T-cell compartment. These data shed further light on functional differentiation within the naive T-cell compartment and the importance of the thymus in naive T-cell homeostasis.

INTRODUCTION

After egression from the thymus, naïve T cells continuously recirculate through the peripheral lymphoid tissues, surveying for their cognate antigen presented by dendritic cells. After priming naïve T cells differentiate into effector/memory T cells that can be divided into subsets based on their phenotype and function. [1] The CD4+CD45RA+CCR7+ naïve T cell compartment has long been regarded as merely a source for effector/memory T cell population without a specific effector function. Naïve CD4+ T cells indeed lack significant production of effector cytokines that define the different T helper subset signatures. However, it has been recently shown that human naïve CD4 T cells have the unique capability of producing large amounts of interleukin-8 (CXCL8), indicating that these cells do have a specific effector function, at least in newborns. [2] How this functional signature relates to naïve T cell dynamics and aging is unknown.

The thymus plays a central role in the generation and maintenance of naïve T cells early in life. After the first year, thymus naïve T cell production starts to decline ending with a very minimal production rate in adults. [3, 4] Even though the adult thymus is still able to produce new naïve T cells, we and others have shown that in contrast to what is seen in mice, most of the naive T cell population in humans is maintained by homeostatic proliferation. [1] Therefore only a marginal decrease of the overall size of the naïve T cell pool is observed during aging. [5-8] Interestingly, new insights indicate that there are also temporal dynamics within the naïve CD4+ T cell pool. Differential expression of CD31 on naïve CD4+ T cells seems to distinguish between a CD31+ subset enriched in recent thymic emigrants (RTE) and a CD31- naïve subset that has most likely arisen after homeostatic proliferation of CD31+ naïve CD4+ T cells. [9, 10] This is apparent in the elderly, where the percentage of CD31+ naïve CD4+ T cells decreases, while the percentage of CD31- naïve CD4+ T cells increases over time. [9-11] Hence, although it is clear that both the thymus and homeostatic proliferation contribute to naïve T cell homeostasis and their relative contribution changes over time, it remains unknown what the functional consequences are of naïve T cell dynamics. Functional changes within the naïve T cell compartment may have major implications for vaccination and immune intervention strategies, especially in the very young and elderly.

A unique model to study human naïve T cell dynamics and function is a group of patients that have undergone neonatal thymectomy during cardiac surgery. In these children, the proportion and number of both CD4+ and CD8+ T lymphocytes are significantly reduced, mainly affecting the naïve T cell population, which is not seen after a thymectomy later in life. [12-16] This seems to result in premature immune aging as the T cell compartment composition and phenotype has been found to resemble that of an adult. [13] Interestingly, 5 to 10 years after neonatal thymectomy many of the thymectomized individuals show signs of thymic tissue regrowth, indicating an unexpected level of thymic tissue regenerative

capacity. [16] It is for now unknown if this thymic tissue regeneration is functional and able to restore the affected naïve T cell compartment and reverse premature immune aging.
We utilized this unique human model of neonatal thymectomy to study naïve T cell dynamics and the role of the thymus in early life, as well as the contribution of thymic regeneration in the functional restoration of the naïve T cell compartment and reversal of premature immune aging. For this purpose a cohort of children early (1-5 years) after neonatal thymectomy as well as a cohort of children older than 10 years of age were studied. Our data suggest that the human naive CD4+ T cell compartment in healthy humans is functionally more heterogeneous than previously thought and that this functional diversity is severely affected by neonatal thymectomy. Furthermore our data provide futher support for the strong regenerative capacity of the thymus, leading to functional restoration of the naïve T cell compartment after neonatal thymectomy

RESULTS

Neonatal thymectomy results in strongly reduced naïve CD4+ T cell percentages and numbers with a shift towards CD31- within the naïve compartment
In line with our previous observations (van Gent et al.), in the first years after neonatal thymectomy (Tx 1-5yr) we observed a significant decrease in absolute CD3+, CD4+, and CD8+ T cell numbers compared to healthy control (HC) (figure 1A). Within the CD4 T cell compartment, the proportion of naïve T cells was significantly reduced after neonatal thymectomy (figure 1B, left panel) and within this naïve T cell population the proportion of recent thymic emigrants (RTE), as measured by CD31+ expression, was also significantly reduced compared to HC (Figure 1B, right panel). Concurrently, an increase in the fraction of both central (Tcm) and effector memory (Tem) CD4+ T cells was present (figure 1C), while the percentage of more differentiated effector memory CD4+ T cells re-expressing CD45RA (Temra) was similar (figure 1C). In absolute numbers both Tem and Tcm were comparable to those in HC while Temra were decreased (suppl. figure 1A). Interestingly, a relative increase (figure 1D) in the recently described self-renewing stem cell memory T cells (Tscm) was noted after neonatal thymectomy, although their absolute numbers still lower compared to HC (suppl. figure 1). Overall, neonatal thymectomy profoundly affected the naïve T cell compartment in numbers and composition and induced a memory skewed CD4+ T cell compartment.

Later in life, thymic tissue regeneration occurs in the majority of children and results in restoration of naïve CD4+ T cell thymic output
A decade or more after neonatal thymectomy (Tx >10yr) absolute CD4 and CD8 T cell numbers were no longer different from older HC (figure 2A). However, percentages of

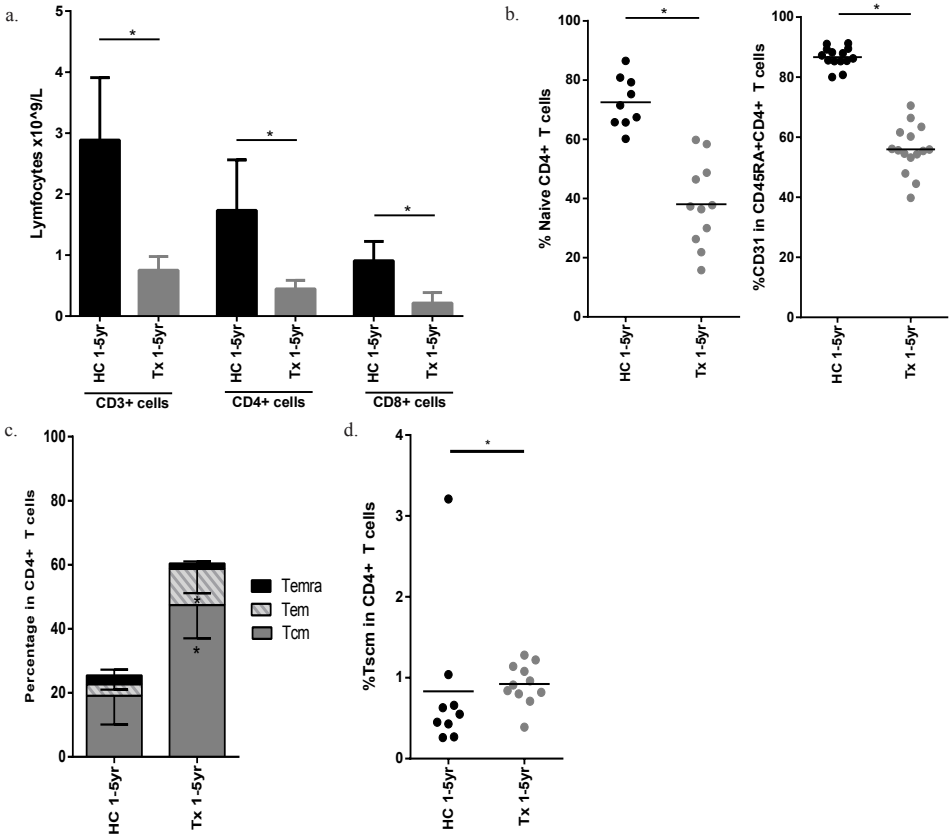


Figure 1. Neonatal thymectomy results in lower naïve CD4+ T cells and skewing towards a memory phenotype in the first years (1-5years) of life

a) Blood lymphocyte count of CD3+, CD4+ and CD8+ T cells in healthy and neonatally thymectomized children for the age groups of 1-5years b) left panel, percentage of naïve T cells (CD45RA+CCR7+) within the CD4+ T cell population; right panel, Percentage of CD31 expressing cells among CD45RA+CD4+ T cells (RTE) c) Percentages of central memory (Tcm, CD45RA-CCR7+), effector memory cells (Tem, CD45RA-CCR7-) and effector memory cells expressing CD45RA (Temra, CD45RA+CCR7-) in CD4+ T cells. d) Percentage of Tscm (stem cell memory T cell, CD45RA+,CCR7+CD28+CD27+FAS+) in CD4+ T cells. Black circles (black bar figure 1a), Young HC (n=9-14); Gray circles (gray bar figure 1a), Young Tx (n=10-15)

naïve CD4 T cells were still significantly lower in the older Tx group (Figure 2B, left panel), especially in a subgroup of patients. When analyzing the percentage of CD31+ within the naïve CD4 T cell compartment we indeed observed a subgroup of Tx patients (open squares, n=7) with a very low percentage of CD31 expressing naïve CD4+ T cells (figure 2B, right panel) in comparison to age-matched HC, suggesting a lack of thymic tissue regeneration within this subgroup. This was indeed confirmed by the absence of thymic tissue on MRI-

scan in these patients (data not shown). These Tx children with low CD31+ RTE also had lower T cell counts (suppl. 2A), a lower proportion of naïve CD4+ T cells (p0.002; 9.0% vs 45%, median, figure 2A) a lower total naïve CD4 T cell count (p<0.001, 27 and 192 x 10^6/L, median, suppl figure 2b) and a concurrent increased proportion of the Tcm compartment (p0.003; 75% vs 47%, median, figure 2c) compared to the Tx individuals with increased CD31 expression. When only focusing on the subgroup of children and young adolescents with normal CD31 percentages in their naïve T cell pool, no significant differences were noted for absolute T naïve and Tscm, Tcm and Tem CD4+ numbers (suppl. figure 2B), as well as the proportion of naïve T cells (figure 2B, right panel), Tscm (figure 2D), and Tem (figure 2C) compared to age-matched HC. Thus, although functional thymic tissue regeneration and restoration of the naïve CD4 T cell compartment after neonatal thymectomy occurs in the majority of children in later life, a subgroup of Tx children shows no signs of restoration of thymic output.

Naïve T cell function is impaired after neonatal thymectomy, but is restored with thymic tissue regeneration in later life

As the naïve CD4+ T cell compartment is greatly altered after neonatal thymectomy, and a distinction between thymic regeneration could be made, we wondered if the functional characteristics of the remaining CD4+ naïve T cells would be affected as well. Following TCR triggering, naïve CD4 T cells normally show strong calcium fluxes, higher than those found in memory T cells ([17] and suppl. Figure 3a). When we compared calcium fluxes following CD3 engagement in naïve CD4 T cells there was a striking significant decrease in the calcium flux in the cells derived from young Tx compared to HC (figure 3a, left and right panel). Since there was no significant difference in peak calcium flux following addition of ionomcyin (suppl. figure 3b) this difference could not be attributed to the differences in maximal fluxing capacity. To translate these findings of lower calcium fluxes to possible functional differences we measured ex vivo cytokine production by the naïve CD4+ T cells. It has been recently reported that naïve T cells from newborns are, in addition to IL-2 production, capable of producing vast amounts of IL-8. In analogy with newborns, we found significant IL-8 production restricted to the naïve CD45RA+CD4 T cells in young HC (Figure 3b). While there was no difference in IL-2 production (Figure 3c), naïve CD45RA+CD4 T cells of young Tx showed a significant decrease in IL-8 production in comparison to age-matched HC (Figure 3d). Together these data indicate an altered function in the early years after neonatal thymectomy.

We next studied whether functionality of the naïve CD4+ T cell was regained with thymic tissue generation later in life. In the older Tx group we indeed found restored calcium fluxes (figure 3e) and IL-8 production of naïve T cells in the majority of children (figure 3f). While the amount of IL-8 production seem sto decrease with age, Interestingly, the capability of naïve CD4+ T cells to produce IL-8 actually was higher in the older Tx group than in the

young Tx group (p<0.000; 6.39% vs 1.46% median, respectively). Persistence of low CD31 expression on CD4+ naïve T cells resulted in persistent low calcium flux (figure 3e), and decreased IL-8 production (p<0.000, 0.73% vs 8.54%, median, figure 3f). Indicating that the thymus is not only necessary for maintaining naïve T cell numbers but also for functional restoration of the naove T cell compartment.

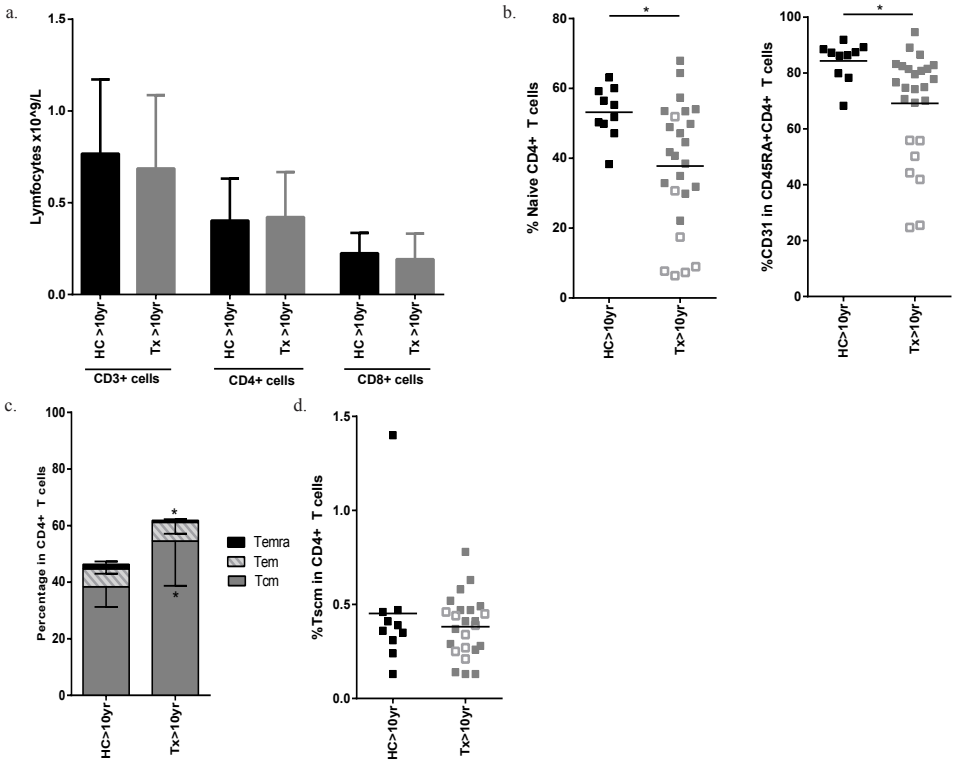
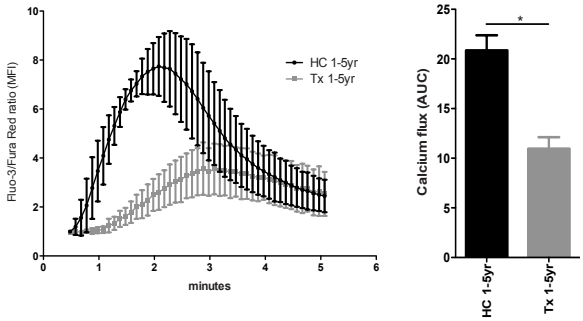


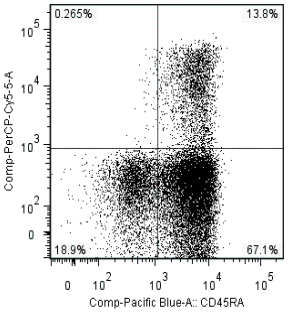
Figure 2. Thymic tissue regeneration results in restoration of the naive and effector T cell compartment later in life

a) Blood lymphocyte count of CD3+, CD4+ and CD8+ T cells in healthy and neonatally thymectomized children older than 10 years of age b) left panel, Percentage of naïve T cells (CD45RA+CCR7+) within the CD4+ T cell population, right panel; Percentage of CD31 expression on CD45RA+CD4+ T cells c) Central memory cells (Tcm, CD45RA-CCR7+), effector memory cells (Tem, CD45RA-CCR7-), and effector memory cells expressing CD45RA (Temra, CD45RA+CCR7-) in CD4+ T cells. d) Percentage of Tscm (stem cell memory T cell, CD45RA+,CCR7+CD28+CD27+FAS+) in CD4+ T cells. Black squares, Older HC (n=10); Gray squares, Older Tx (n=24-26); gray open squares, Older Tx with low RTE expression (n=7) Depicted mean (±SD), *P<0.05,

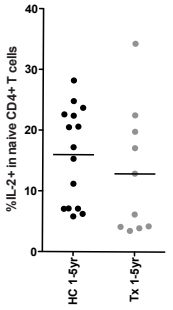
a.


Fluo-3/Fura Red ratio (MFI)
minutes
HC 1-5yr
Tx 1-5yr
Calcium flux (AUC)
*
HC 1-5yr
Tx 1-5yr

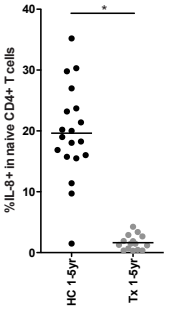
b.


0.265%
13.8%
18.9%
67.1%
Comp-PerCP-Cy5-5-A
Comp-Pacific Blue-A:: CD45RA

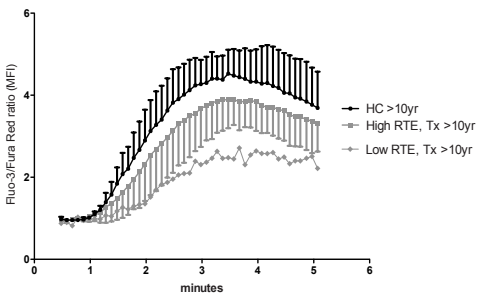
c.

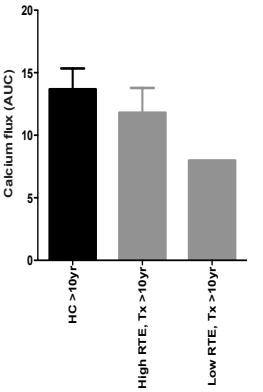

%IL-2+ in naive CD4+ T cells
HC 1-5yr
Tx 1-5yr

d


%IL-8+ in naive CD4+ T cells
*
HC 1-5yr
Tx 1-5yr

e.


Fluo-3/Fura Red ratio (MFI)
minutes
HC >10yr
High RTE, Tx >10yr
Low RTE, Tx >10yr


Calcium flux (AUC)
HC >10yr
High RTE, Tx >10yr
Low RTE, Tx >10yr

f.

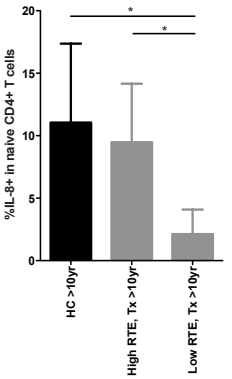

%IL-8+ in naive CD4+ T cells
*
*
HC >10yr
High RTE, Tx >10yr
Low RTE, Tx >10yr

Figure 3. Naïve CD4+ T cells are impaired after neonatal thymectomy in early life, but restore after thymic regeneration in later life
a) left panel, calcium flux of CD4+ naïve T cell in young children (Young HC, n=4 black line, young Tx, n=6 gray line, mean +/-SD). Right panel, area under the curve (AUC) of the calcium flux b) representative dotplot of IL-8 production in CD45RA+ CD4+ T cell population of young HC c) IL-2 production in naïve CD4+ T cells of young HC (n=15) and young Tx patients (n=10) d) IL-8 production in naïve CD4+ T cell of young HC (n=19) and youbg Tx patients (n=15) e) left panel, calcium flux of CD4 naïve T cell of older HC and Tx children. Right panel, AUC of the calcium flux; (Older HC, n=5, Older Tx, n=6, Older Tx, with low RTE, n=1) f) IL-8 production in naïve CD4+ T cells of older HC (n=10) and older Tx patients (Older (high RTE) Tx, n=17; low RTE, n=7). Data sepicted mean (±SD)

Neonatal thymectomy induces the loss of IL-8 expression within the CD31+ naïve CD4+ T cell compartment

The naïve T cell compartment can be divided by CD31 expression, but little is known about the functional differentiation between the CD31 positive and negative naïve CD4+ T cell subset. In young Tx children the percentage of CD31+ CD4+ naïve T cells within CD4+ T cells was lower, while CD31- naïve CD4+ T cells was significantly increased compared to healthy children (figure 4a). In young healthy children, the production of IL-8 was almost exclusively due to the CD31+ naïve CD4+ T cell compartment (fig 4b, black dots). Interestingly, IL-8 expression within this subset was significantly lower young Tx children (figure 4b), with a concomitant increase in IFNγ expression. In the CD31- naïve fraction IFNγ_expression was further increased in both the HC and Tx group in comparison to the CD31+ naïve CD4+ T cells (suppl. figure 4a). In older Tx patients, restoration of functional thymic tissue resulted in the restoration of IL-8 production by CD31+ naïve CD4+ T cells, while in patients with low RTE no such restoration was seen (figure 4c). Together these data indicate that IL-8 production by naïve CD4+ T cells is not diminished due to a lower number of CD31+ naïve CD4+ T cells, but by lowered intrinsic IL-8 production of CD31+ naïve CD4+ T cells and is dependent on (recent) thymic output.
When we analyzed IL-8 expression in single positive (SP) CD4+ thymocytes we found very low production of IL-8 compared to their peripheral naïve CD4+ T cell counterpart (figure 4d), suggesting that IL-8 production is initiated shortly after egress from the thymus. The expression of PTK7 on CD31+ naïve CD4+ T cells has been identified as a marker of recent thymic emigrants that have very recently left the thymus. [18] While these cells have been regarded as quiescent, we found a significant enrichment of IL-8 production within the PTK7-positive fraction of peripheral CD31+ naïve CD4+ T cells (figure 4e, suppl. figure 5a). To further assess the correlation of IL-8 production by PTK7+ RTE we stimulated CD31+ naïve T *in vitro* with a cocktail of cytokines that are known to decrease PTK7 expression. [18] With each cell division, a decrease of PTK7 expression was observed with a concomitant decrease in IL-8 production (figure 4f and 4g, suppl. figure 5b and 5c). At the same time, expression of CD31 remained stable after cytokine stimulation, with an increase

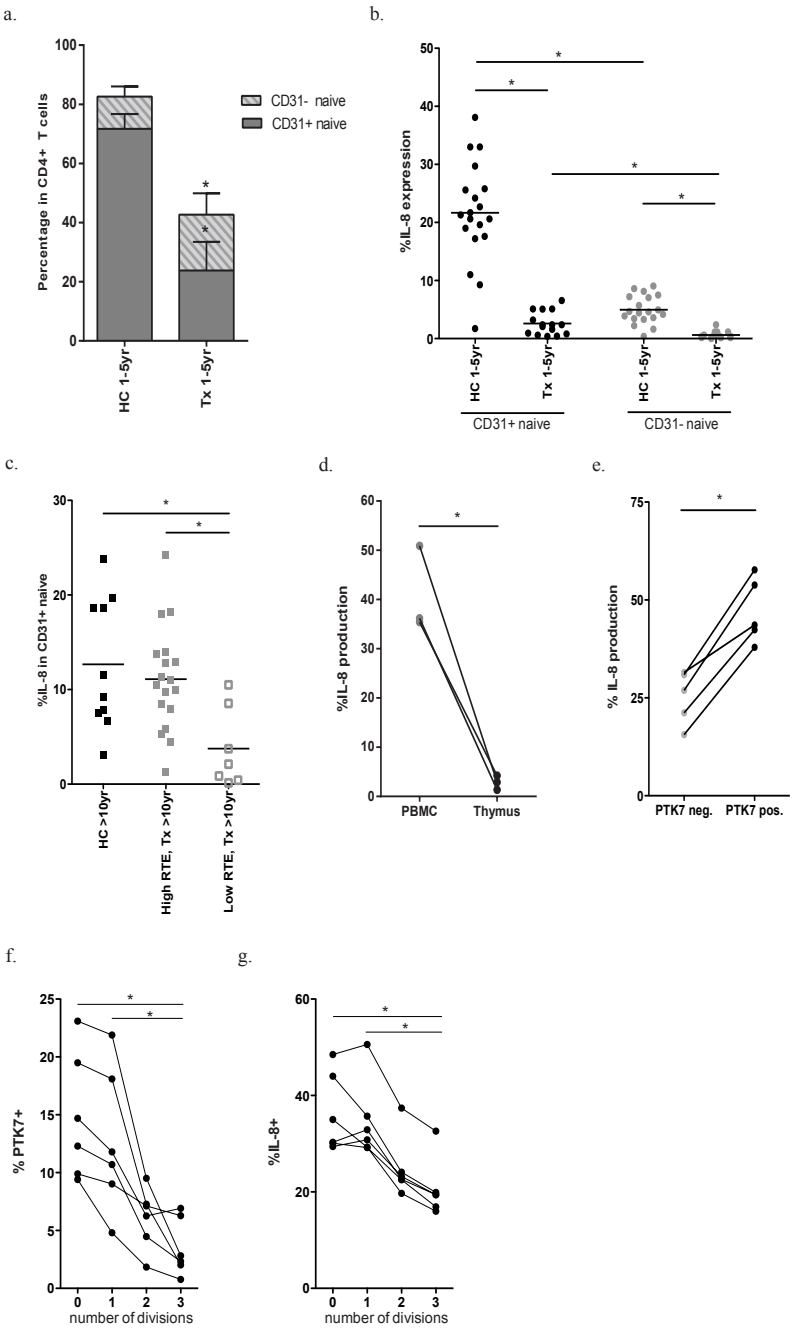


Figure 4. IL-8 production is enriched in the PTK7+ RTE fraction of CD31+ naïve CD4+ T cells and lost after cell division.

a) Proportion of CD31+ and CD31- naïve CD4+ T cells in the CD4+ T cell compartment of HC 1-5yr (n=19) and Tx 1-5yr (n=15) b) expression of IL-8 by CD31+ and CD31- naïve CD4+ T cells of young HC

(n=19) and Tx patients (n=15) c) expression of IL-8 by CD31+ and CD31- naïve CD4+ T cells of older HC (n=10) and older Tx patients separated on the basis of low (n=7) or high percentage of CD31+ (n=19). d) Paired IL-8 production measurements by single positive (SP) CD3highCD4+CD8- thymocytes and blood CD31+ naive CD4+ T cells (PBMC) from the thymectomized neonates (n=3) e) IL-8 production by PTK7+ (black dots) and PTK7- (gray dots) CD31+ naïve CD4+ T cells from young HC (n=5) f) PTK7 expression after each cell division following cytokine stimulation of FACSsorted CD31+ naïve CD4+ T cells from older HC (n=6) g) IL-8 expression after each cell division following cytokine stimulation of FACSsorted CD31+ naïve CD4+ T cells from older HC (n=6). Data depicted mean (±SD)

in IFNγ expression per cell division (Suppl. figure 5d and 5e respectively). Overall, our data indicate that IL-8 production is predominantly enriched in the PTK7+ fraction of the CD31+ naïve T cell compartment and that this functional heterogeneity within the CD31+ naïve CD4+ T cell compartment is cell division related.

CD31+ naïve CD4+ T cells of thymectomized children are distinct from those of healthy controls and resemble their CD31- naïve CD4+ T cell counterpart

To further asses any transcriptional differences between naïve T cells of healthy controls and thymectomized children we performed performed gene expression profiling by RNA sequencing. We analyzed the transcriptome of both FACSsorted CD31+ and CD31- naïve CD4+ T cells from thymectomized children (n=4, between 1 and 5 years after Tx) and age-matched healthy children (n=3). Unsupervised cluster analysis showed that both CD31+ and CD31- naïve CD4+ T cell subsets of healthy children did not cluster with their counterparts in thymectomized children (Figure 5A). While within the group of young HC no specific clustering was noticed regarding both naïve T cell subsets, in young thymectomized children both the CD31+ and CD31- naïve subsets clustered together within the same patient (Figure 5A, 5B). Thus CD31+ naïve T cells of thymectomized children now resembled their CD31- naïve counterpart, indicating loss of heterogeneity. However, both naïve subsets of thymectomized children were still distinct from the CD31+ and CD31- naïve T cell subsets of healthy controls and especially memory T cells of either patient group (Figure 5B and data not shown). This suggests that both naïve T cell subsets may undergo transcriptional changes after thymic removal leading to converging transcriptional profiles.

Transcript analyses resulted in 305 targets that differed between CD31+ naïve CD4+ T cells of thymectomized and healthy children (Suppl. table 1). In contrast, only 2 other transcripts (MAF and FAM129C), next to CD31 (PECAM-1) were found to differ between CD31+ and CD31- naïve CD4+ T cells of thymectomized children (Suppl. table 2). This further demonstrates that CD31+ naïve CD4+ T cells after neonatal thymectomy resembled CD31- naïve CD4+ T cells despite expression of CD31 on naïve CD4+ T cells. Together, these data indicate that there is transcriptional differentiation within the naïve CD4 T cell compartment, which is lost following neonatal thymectomy.

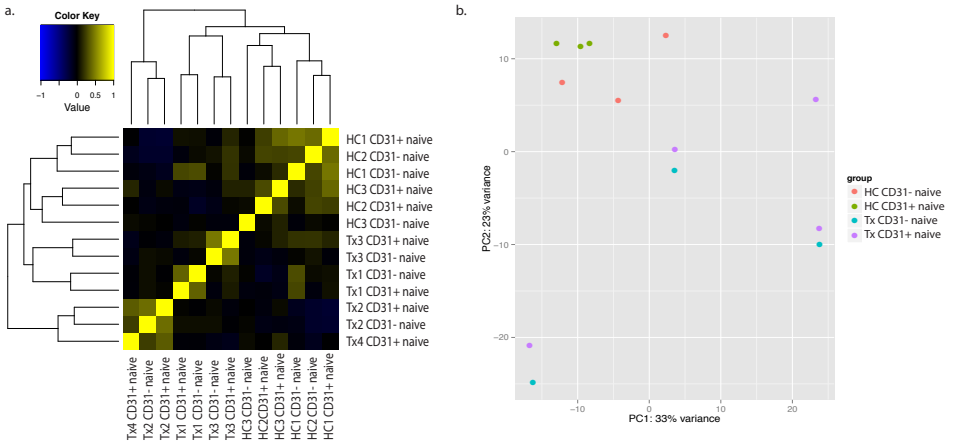


Figure 5. Functional differentiation within CD31+ naïve CD4+ T cells is lost after neonatal thymectomy. a) Correlation clustering of CD31+ and CD31- naïve CD4+ T cells of young HC (n=3) and young Tx (n=4), yellow indicating high positive correlation, while blue high negative correlation b) Principal component analysis of CD31+ and CD31- naïve T cells of young HC (n=3) and young Tx (n=4).

DISCUSSION

Naïve CD4 T cells are the source of the effector/memory T cell compartment and are considered relatively homogeneous as opposed to the effector/memory T cell population. To stay truly naïve, naïve T cells have to avoid differentiation and remain relatively quiescent. With human aging thymic output and naïve CD4 T cell numbers decline but it has not been clear whether this is accompanied by (functional) differentiation of naïve T cells and loss of true naivety. By utilizing a unique cohort of neonatally thymectomized children we here demonstrate functional differentiation within the human naïve CD4 T cell compartment, which is lost following neonatal thymectomy. In addition, functional thymic regeneration was seen in the majority of neonatally thymectomized patients and resulted in restoration of functional differentiation and rejuvenation of the naïve CD4+ T cell compartment.

Throughout life, the peripheral T-cell pool is maintained at a relatively constant size. As thymic export stops or diminishes, the maintaining peripheral T-cell pool will proliferate, replenishing the peripheral space and gradually acquiring an effector/memory phenotype, as also seen in lymphopenic conditions and aging. [7, 19, 20] This slowly results in the loss of naïve T cells, and skewing towards the effector/memory phenotype. After neonatal thymectomy, we detected an increase in proliferation of T cells as well as skewing towards a central memory (Tcm) and effector memory (Tem) phenotype, at the expense of naïve T cells. Of interest, an increased proportion of a newly reported T cell subset, called T memory

stem cells (Tscm), is seen after neonatal thymectomy. These Tscm are suggested to be long-lived and endowed with differentiation potential and self-renewal abilities, as they can generate effector/memory T cell subsets while preserving the original naïve-like phenotype. [21] Recent human in vivo analysis suggest that relative Tscm frequencies do not vary much with age and constitutes a stable fraction, but could increase to compensate for reduced naïve T cell output. [22] The increased Tscm compartment after neonatal thymectomy could therefore be compensatory for the reduced naïve T cell compartment and aid in the expansion of the CM and EM T cell compartment to normal levels. Its actual contribution is however uncertain as Tscm numbers are decreased in thymectomized children despite their increased proportion in CD4+ T cells.

During normal T cell activation, T cell antigen receptor (TCR) ligation results in rapid cytoskeletal rearrangements and a dramatic increase of intracellular calcium (Ca(2+)) concentration. [23] Thus, Ca(2+) signaling is essential for T cell activation and T cell-dependent immune responses. After neonatal thymectomy, a drastic decrease in calcium flux is seen. Not only is the height of the flux decreased, the calcium flux also seems to be delayed in comparison to healthy controls. The potential for an adequate calcium flux was however not affected, as stimulation with ionomycin resulted in an equal maximum calcium peak. In addition, T cell activation dependent production of IL-2 was also not diminished, demonstrating that despite an altered calcium flux phenotype naïve T cells were still able to respond. A delayed calcium flux response in naïve CD4+ T cells is not only apparent after neonatal thymectomy but also with increasing age. [17] This delay was not due to an increased proportion of CD31- naïve T cells; even though these cells do show lower calcium flux compared to the total CD4+ naïve T cell population, their calcium response was not delayed (data not shown). Together these data indicate features indicative of premature aging and functional differentiation within the naïve T cell compartment after neonatal thymectomy.

Another indication of premature aging of the naïve T cell compartment was the loss of IL-8 production that we observed in naïve T cells of thymectomized children. IL-8 production has recently been revealed as a signature effector function of naïve/immature CD4+ T cells in newborns. We here showed that IL-8 producing naïve T cells are also present in infants but gradually decline with age. The almost complete loss of IL-8 and concomitant increase in IFNγ production of naïve CD4+ T cells after neonatal thymectomy indicate a functionally altered naïve T cell compartment, resembling that of the adult immune system. While the production of IL-8 of CD31+ naïve CD4+ T cells was strongly dependent on thymic presence, SP CD4+ thymocytes barely produced any IL-8. When we further subdivided the CD31+ naïve population based on PTK7 expression we found an enrichment of IL-8 positive cells within the PTK7+ CD31+ naïve RTE CD4+ T cells. Together this suggests that there is a very small window of differentiation in which IL-8 expression is induced. The loss of IL-8 expression could be induced by a mixture of cytokines that also

downregulates PTK7 [18], while maintaining CD31 expression, indicating that non-antigen driven proliferation is sufficient to induce functional differentiation of RTE.
CD31 has been shown to identify a compartment enriched in recent thymic emigrants, whereas CD31- naïve T cells are thought to arise following homeostatic proliferation.[9,10] When comparing gene expression profiles of CD31+ naïve CD4+ T cells of both healthy and thymectomized children large differences were found (>200 genes differentially expressed). More importantly, transcriptional analysis of CD31+ and CD31- naïve T cells from thymectomized children revealed an almost complete overlap between the two populations, which was not present in healthy children. This demonstrates that after neonatal thymectomy CD31+ T cells undergo differentiation towards CD31- cells without the loss of CD31. The phenomenon of partial differentiation of naïve T cells in aging has also been suggested based on differential expression of miRNAs (miR-181a, miR146a and miR-21) [24] and CD25 expression on naïve CD4+ T cells. [25] Hence with aging naïve T cells may acquire features of memory cells with maintenance of their naïve phenotype. This partial differentiation may have detrimental consequences because the naïve T cells seem to have lost what is essential for their function, while not fully having gained their new differentiation state.
We have previously reported that for a majority of the children evidence of thymic tissue regeneration is observed after 5-10 years post-thymectomy.[16] It is however unknown if this regeneration of thymic tissue is functional and able to restore the thymectomy related immune disturbances. We assessed thymic tissue regeneration and thymic output by MRI imaging and RTE (% CD31 in naïve CD4+ T cells) measurements. In a previous cohort a similar proportion of children showed regeneration of thymic tissue assessed by MRI-scan.[16] However, the presence of thymic tissue does not have to indicate a functional thymus as well as lack of thymic tissue cannot always be adequately determined. We therefore assessed functional thymic tissue regeneration primarily via RTE measurement. In our cohort, all patients with lower RTE did not show any thymic tissue on MRI, while only a small portion of children with higher RTE did not present evident thymic tissue on MRI. Thymic tissue regeneration, assessed by RTE, resulted in a phenotypical restoration of the naïve T cell compartment, as well as functional restoration of the calcium flux and neonatal effector cytokine production of IL-8. In a quarter of the children no evidence for functional thymic tissue regeneration (low RTE) was detected after at least a decade. These children continued to have a disturbed phenotypical and functional naïve/effector compartment, comparable to children in their first years post-thymectomy. The rejuvenation capacity of the thymus is an interesting option to explore and many interventions have tried to restore thymic function in the elderly or in a setting of hematopoietic stem cell transplantation. Although to date no interventions fully restore thymic function in the aging host, systemic administration of various cytokines and hormones or bone marrow transplantation have resulted in a degree of increased thymic activity and T-cell output with age. [26, 27 28 29]

Why in some children thymic tissue regenerates and not in others is unclear and warrants further investigation. We previously hypothesized that the age of thymectomy might play a role in the regenerative capability later in life, as some studies with children thymectomized at an average of 2.6 years still reported diminished naive T-cell counts and naive T-cell TREC contents well into the third decade of life. [12] In the current cohort all children were thymectomized within the first month of life, but resulted in a similar proportion of thymic regeneration as our previous cohort in which most (87%) children were thymectomized within 4 months of life.[16] Besides the age of thymectomy or possible differences in soluble factors stimulating thymic regeneration it could be that the extent of thymic removal was so rigorous in these children that no thymic tissue remained to serve as a platform for regeneration.

In conclusion, this study shows that the thymus has a detrimental role in the phenotypical and functional maintenance of the naïve CD4+ T cell population and is able to functionally regenerate later in life. We propose that thymic tissue should be preserved as much as possible during cardiac surgery, as not all children regenerated thymic tissue. Additional, these findings give further insight in the functional heterogeneity of naïve T cell compartment by identifying differential IL-8 production within the CD31+ naïve CD4+ T cell compartment and the dependence of this differentiation on thymopoiesis. This appreciation could aid us in better monitoring thymopoiesis, as CD31+ expression can persist on naïve T cells even though they may be partially differentiated, and further understanding the mechanisms of naïve T cell homeostasis after for instance immune ablative therapy, as well as in aging.

METHODS

Study population

Patients who had undergone complete thymectomy within the first month of life because of surgery to treat congenital heart defect at the Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands, were included in this study. Surgery involving the major vessels, such as transposition of the great arteries, hypoplastic left heart syndrome, and hypoplastic arch with or without coarctation of the aorta routinely necessitates thymectomy. An overview of the study population is described in table 1.

Blood samples were taken between 1 to 5 years (referred to as 'young Tx') and after 10 years (referred to as 'older Tx') of neonatal thymectomy since we have previously shown that the potential to regenerate thymic tissue occurs at approximately 5-10 years. [16] Patients showed no clinical signs of infection at time of inclusion nor had a syndrome or genetic disorder (e.g. 22q11 deletion, trisomy 21). A healthy age- and gender matched control group (HC) who underwent elective surgery were also included. The children were considered

immunologically healthy because they did not have a history of infectious diseases or a hematologic or immunologic disorder. Written consent was obtained from all study participants or their legal guardians.
The study was approved by the medical ethical committee of the University Medical Center Utrecht and written consent was obtained from all study participants or their legal guardians in agreement with Helsinki Declaration of 1975, revised in 1983.

Visualization of thymic tissue after thymectomy

To determine whether the thymus remained absent >10 years after neonatal thymectomy, the presence of thymic tissue in patients was evaluated during follow-up. The presence or absence of thymic tissue on MRI scans was evaluated and scored by an experienced pediatric radiologist.

Cell preparation and flow cytometry

PBMCs were isolated from heparinized blood samples by using the Ficoll Isopaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden), and viably frozen and stored in liquid nitrogen until further processing. Characterization of the T-cell compartment was performed on thawed cryopreserved PBMCs that were washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FCS and 0.1% sodium azide) and blocked with normal mouse and rat serum. The cells were incubated in 25 μL FACS buffer containing the appropriately diluted antibodies. For intracellular staining of Ki-67, the cells were first surface stained, followed by fixation and permeabilization according to the manufacturer's protocol. Antibodies against human CD8 (Sk-1) CD31 (WM59), CD28 (L293), CD27 (L128), FAS (CD95, clone:DX2), KI-67 (B56) were from BD Biosciences (San Jose, CA), from R&D systems (Minneapolis, MN), and CD3 (UCHT1), CD4 (RPA-T4), CD45RA (HI100), from Biolegend (San Diego, CA). Finally, stained mononuclear cells were washed twice in FACS buffer and run on an FACS Canto II and analyzed by using FlowJo software (Treestar).

Table 1. Patient characteristics

	Young HC	Young Tx	Older HC	Older Tx
Number of patients	19	17	11	26
Age at thymectomy (days)	-	8 (7.93)	-	7 (3.92)
Age at blood draw (years)	2.04 (1.00)	2.12 (1.16)	13.70 (1.48)	15.89 (4.16)
Gender (Male)	13 (68.4%)	11 (64.7%)	7 (63.6%)	17 (65.4%)

Data is shown as median (±SD) for 'age at of thymectomy' in days and 'age at blood sampling' in years Number (percentage) is shown for male gender

As cell counts and/or sufficient cells were not available for all samples due to the limited amount of blood allowed to be taken from children, some data points are not shown for all study subjects.

Calcium Flux

T-cells were purified from fresh PBMCs by magnetic-bead separation using the biotin human T-lymphocyte enrichment cocktail (BD Imag, BD Biosciences) according to manufacturer's instructions. Purity of MACS-sorted CD3+ T-cells was >90%.

500.000 MACS-isolated T-cells were incubated with in 0.2mg/ml Fluo-3, 0.4mg/ml Fura Red (Invitrogen, Carlsbad, CA) for 30minutes at 37C and during the last 10 minutes a combination of the FACS antibodies CD3 (OKT3, Biolegend), CD8 (SK1, BD Bioscience), CD45RO (UCHL1, Biolegend) was added. Cells were washed twice and resuspended in Hank's balanced salt solution (HBSS) supplemented with fecal calf serum (FCS). Baseline cytosolic calcium levels were measured on a FACS Canto II flow cytometer. Next, 20ug/ml anti-IgG $F(ab')_2$ fragments (Jackson Immunoresearch, West Grove, PA) were added and calcium flux was measured for 4,5 minutes. Subsequently, 0.1mg/ml ionomycin (Caliochem, San Diego, CA) was added. Calcium flux of CD4+ naïve T cells was assessed by gating on CD3+ CD8- CD45RO- T cells, and of memory CD4+ T cells on CD3+CD8-CD45RA- T cells (CD45RA (HI100, Biolegend)), as binding of anti-IgG F(ab')2 fragments on other fluorochromes other than CD3 could potentially interfere with the calcium flux.

T-cell stimulation

200.000 PBMC were stimulated with PMA (20ng/ml) and ionomycin (1ug/ml) for 5 hours at 37C, 5% CO_2 in culture medium (10% hAB-serum 1% penicillin, streptomycin and 1% L-glutamin). After 1 hour of culture Golgistop (BD) was added to the culture. Cells were then washed, blocked with normal mouse serum followed by extracellular staining, fixation in Cytofix/Cytoperm and washing in Perm/Wash solution (Cytofix/perm kit, BD). Finally, cells were blocked with normal rat serum and incubated with the appropriate mAbs against IL-2 (MQ1-17H12, BD Biosciences) and IL-8 (BH0814, Biolegend) for intracellular cytokine staining.

In the case measurement of IL-8 production in thymus (SP CD4+ Thymocytes, $CD3^{high}$CD4+CD8-) and PBMC (CD31+CD45RA+ CD4+ T cells), samples were paired from the same neonate. Thymocytes were isolated via straining it through a mesh filter, followed by via Ficoll Isopaque density gradient centrifugation. PMA/ionomycin stimulation was performed as described above and on fresh isolated cells.

In vitro cytokine stimulation

CD31+ naïve T cells were FACSsorted (supplemental figure 6) and stained with Cell Tracer Violet (final concentration 2uM) for 7 minutes at 37C. The reaction was stopped by adding 10x volume of cold, filtered 100% fetal calf serum (FCS). Cells were washed and plated at 50.000 T cells per well in culture medium. Followed by stimulation with 25ng/ml of recombinant human IL-6 (BD Pharmingen), IL-7 (ImmunoTools), IL-10 (ImmunoTools), IL-15 (ImmunoTools) and IFNγ (eBioscience) as previously described, for 6 days. [18] After cytokine stimulation, cells were washed and stimulated with PMA and ionomycin as described in the 'T-cell stimulation' paragraph to determine IL-8 and IFNγ cytokine production. PTK7 (Miltenyi Biotec, 188B), and isotype staining (Miltenyi Biotec, S43.10) were performed to assess PTK7+ cells, in addition to CD3 CD4 CD31 and CD45RA

RNA sequencing

PBMC from neonatally thymectomized children (1-5years of age, n=4) and age-matched controls (1-5years, n=3) were FACSsorted for on CD31+ and CD31- naïve CD4+ T cells using the following mAbs; CD3 (Clone OKT3, Biolegend), CD4 (Clone:RPA-T4, eBioscience), CD25 (Clone: BC96, Biolegend), CD45RA (HI100, Biolegend) CD45RO (Clone: UCHL1, Biolegend), CD31 (Clone WM59, Biolegend), CD127 (Clone: eBioRDR5, eBioscience), CCR7 (Clone:150503, R&D Systems). To ensure there is no contamination of regulatory T cells and Temra occurred, these were cells excluded, according to the gating strategy depicted in supplemental figure 6. Sorted cells were lysed in RLTplus buffer with (1%) beta mercaptoethanol.

Total RNA was purified from sorted CD31+ and CD31- naïve CD4+ T cells using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen) as according to manufacturer's instructions. RNA was stored at -80C until further processing. mRNA was isolated using the Poly(A) Beads (NEXTflex). Sequencing libraries were prepared using the Rapid Directional RNA-Seq Kit (NEXTflex) and sequenced on Illumina NextSeq500 to produce single-end 75 base long reads (Utrecht DNA sequencing facility). Sequencing reads were mapped against the reference genome (hg19 assembly, NCBI37) using BWA [30] package (mem –t 7 –c 100 –M –R). Reads mapping to multiple locations were excluded from the analysis. The RPKM values and the raw read numbers were calculated using the rnaseq_ countgeneread function from Cisgenome v2. [31]

Data analysis and statistics

Statistical significance between two groups was assessed using the Mann-Whitney U test for unpaired data and Wilcoxon signed rank test for paired data. Statistical difference is indicated with * for $P<0.05$. In the case of the cytokine stimulation of CD31+ naïve CD4+ T cells, Kruskall-Wallis test was performed and comparisons were considered significant at <0.05 after Dunn's multiple comparison test.

Differentially expressed genes were identified using the DESeq2 package with standard settings. [32] Genes with log2 fold change larger than 0.6, base Mean >10 and p-value adjusted < 0.05 were considered as differentially expressed. The unsupervised heatmap clustering was performed with the quantile normalized and median centered RPKM values using the heatmap.2 function in R function with the standard clustering settings. Only the transcripts with the minimal expression larger than 4 RPKM and at least 2-fold difference in RPKM values between the values at the 20^{th} and 80^{th} percentile were used for the clustering. In the correlation heatmap, the Pearson's correlation was used as an index of similarity.

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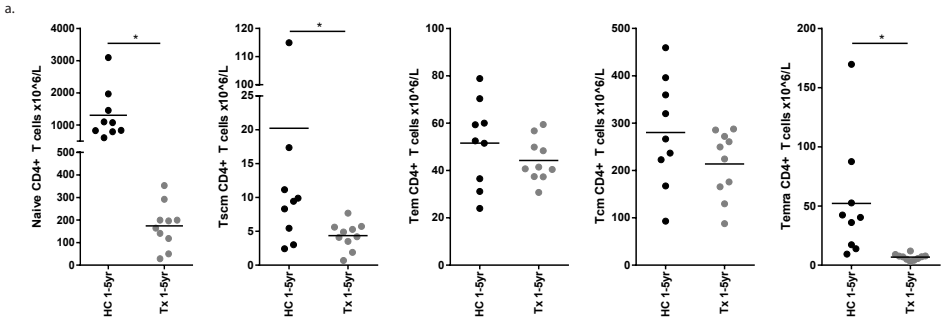
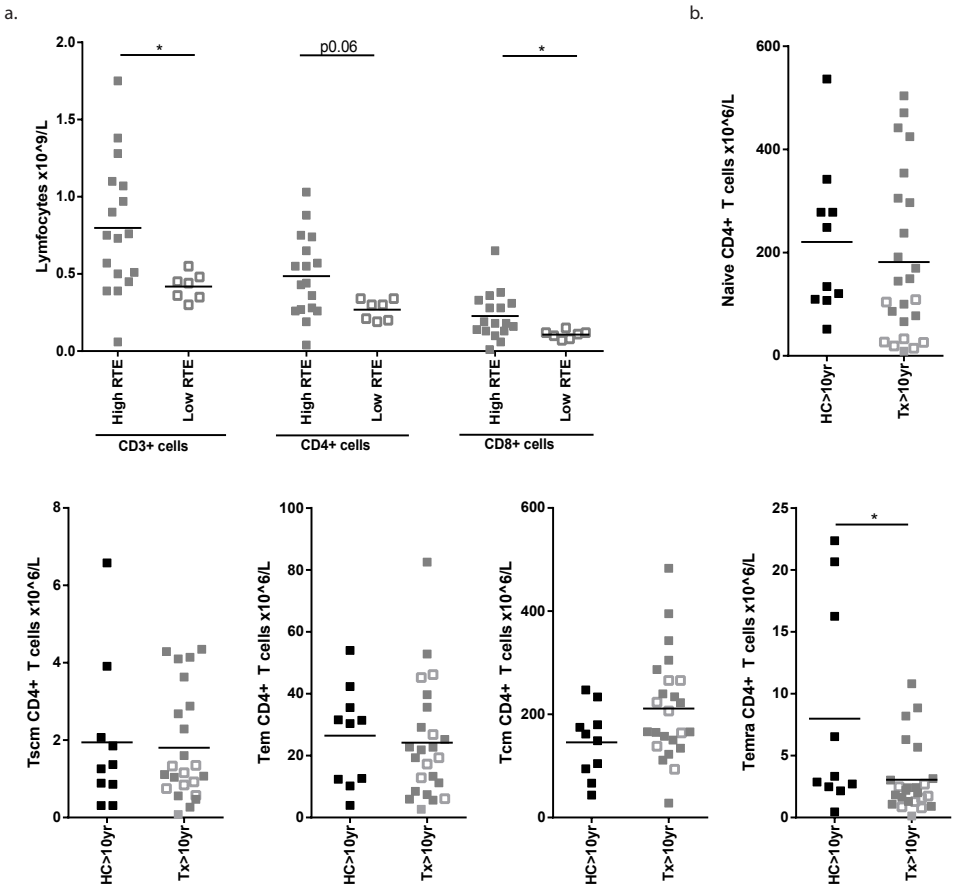
SUPPLEMENTAL INFORMATION

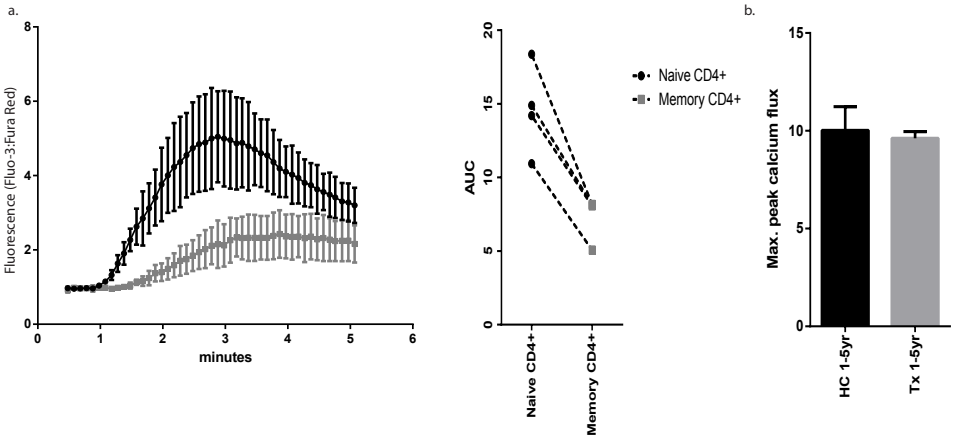


Supplemental figure 1. Absolute cell count of naïve/memory compartment and selection of Tscm cells. Absolute cell number of naïve (CD45RA+CCR7+), Tscm (Stem cell memory T cell, CD45RA+CCR7+CD28+CD27FAS+), central memory (CM, CD45RA-CCR7+), effector memory (EM, CD45RA-CCR7-), and Temra (effector memory re-expressing CD45RA, CD45RA+CCR7+) CD4+ T cells respectively (young HC, n=9, black circles; young Tx, n=10, gray circles), *P<0.05.

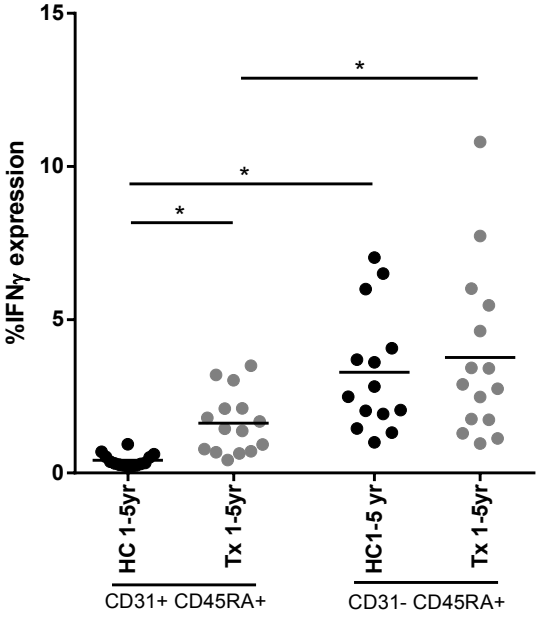


Supplemental figure 2. Thymic regeneration occurs in the majority of children and restores the naïve T cell compartment.

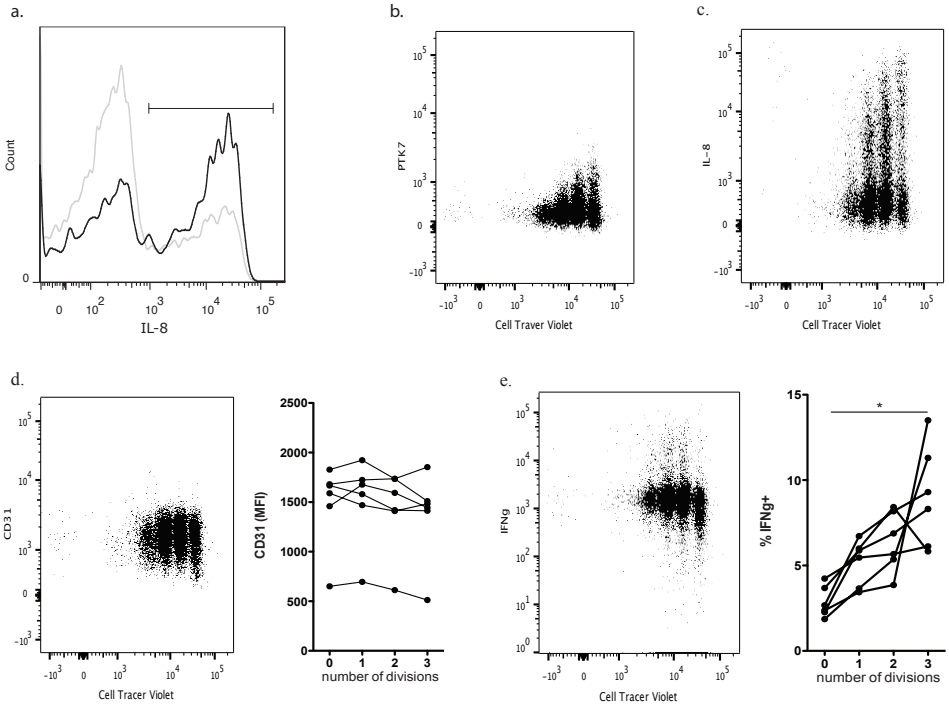
a) Absolute cell number of CD3+, CD4+ and CD8+ T cells (lymphocytes x 10^9/L) in neonatally thymectomized children, aged 10 years or older, divided by high RTE expression (gray squares, n=17) and low RTE expression (black squares, n=7) **b)** Absolute cell number of naïve (CD45RA+CCR7+), Tscm (Stem cell memory T cell, CD45RA+CCR7+CD28+CD27FAS+), central memory (CM, CD45RA-CCR7+), effector memory (EM, CD45RA-CCR7-), and Temra (effector memory re-expressing CD45RA, CD45RA+CCR7+) CD4+ T cells respectively (Older HC, n=10, black squares; Older Tx, n=24, gray squares) *P<0.05.



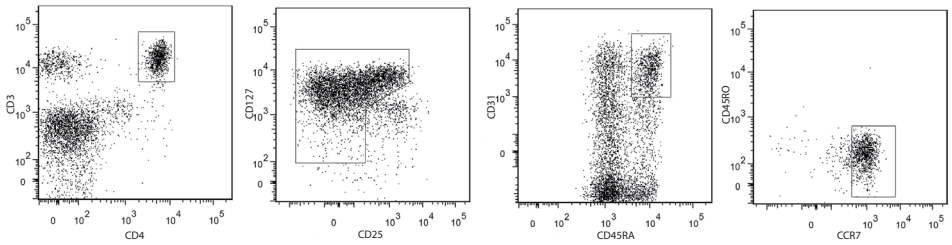
Supplemental figure 3. CD4+ naïve T cells of young thymectomized children show lower calcium flux and IL-8 intensity, but have similar calcium flux capacity as healthy children.
a) left panel, Calcium flux of older HC naïve CD4+ (black line, n=4) and CD4+ memory (gray line, n=4); right panel, AUC of paired calcium flux of naïve and memory CD4+ T-cells b) Maximum calcium flux after stimulation with ionomycin (HC 1-5yr, n=4; Tx 1-5yr, n=6)



Supplemental figure 4. CD31- naïve CD4+ T cells have increased IFNγ production IFNγ production of CD31+ and CD31- naïve CD4+ T cells in young healthy and thymectomized children. (HC 1-5yr, n=15-19, black circles; Tx1-5yr, n=15, gray circles)



Supplemental figure 5. Production of IL-8 by CD31+ naïve CD4+ is correlated to PTK7.
a) Representative histogram of IL-8 production in PTK7+ (black line) and PTK7- (gray) CD31+ naïve CD4+ T cells. Geo mean of PTK7 expression in IL-8 negative (gray) and IL-8 positive (black) CD31+ naïve CD4+ T cells (n=5; HC 1-5yr). b) representative FACSplot of PTK7 expression of each cell division after cytokine stimulation. c) representative FACSplot of IL-8 expression of each cell division after cytokine stimulation. d) representative FACSplot of CD31 expression after cytokine stimulation per cell division. Right panel, summary of mean fluorescence intensity (MFI) of CD31 per cell division after cytokine stimulation e) left panel, representative FACSplot of IFNγ expression after cytokine stimulation per cell division. Right panel, summary of IFNγ percentage per cell division after cytokine stimulation



Supplemental figure 6. Representative FACS gating strategy for sorting of CD31+ naive CD4+ T-cells. Single cell lymphocytes were gated on CD3+ and CD4+ expression (1st panel), followed by exclusion of CD25+CD127- (Treg) cells (2nd panel), selection of CD31+CD45RA+ expression (3rd panel), and further exclusion of CD45RO+ and CCR7- T cells to exclude possible Temra or CD45RA+CD45RO+ double positive T cells contamination (4th panel)

Supplemental table 1. Differential gene expression between CD31+ naïve CD4+ T cells of thymectomized and healthy children.

Gene name	Refseq id	Base Mean	log2FoldChange	p-value	p-value adjusted
GBP5	NM_001134486	1978,040041	2,37934647	3,31901E-18	2,94496E-14
BHLHE40	NM_003670	435,0652588	2,522295576	2,64317E-18	2,94496E-14
GBP5	NM_052942	2012,135246	2,358755368	1,37768E-17	8,14943E-14
EDA	NM_001005609	72,89280168	2,862187447	6,69756E-16	2,3771E-12
EDA	NM_001399	72,89280168	2,862187447	6,69756E-16	2,3771E-12
TOX	NM_014729	172,1769054	-2,216897273	1,22234E-15	3,61527E-12
MYO15B	NR_003587	2588,323744	-1,511958569	2,5154E-15	6,3769E-12
PTGER2	NM_000956	508,8306362	2,377058453	4,91551E-13	1,09038E-09
PREX1	NM_020820	1952,886091	1,688756899	8,57843E-13	1,69148E-09
AFF2	NM_002025	112,8215466	-2,40748195	5,09039E-12	9,03341E-09
RICS	NM_001142685	475,3733472	-1,314923782	3,92632E-10	6,33423E-07
FAM101B	NM_182705	2808,041541	-0,927702667	9,50437E-10	1,29742E-06
ISM1	NM_080826	98,61617992	2,163118812	8,83679E-10	1,29742E-06
SUSD4	NM_017982	656,10292	1,553012575	2,62934E-09	3,33287E-06
TIMP1	NM_003254	592,6183492	1,633836055	4,11067E-09	4,8632E-06
RICS	NM_014715	459,7463145	-1,273561189	4,61642E-09	5,12019E-06
CACNA1I	NM_021096	658,4877087	1,564584366	2,58998E-08	2,55344E-05
CACNA1I	NM_001003406	658,4877087	1,564584366	2,58998E-08	2,55344E-05
HES4	NM_001142467	114,9469605	1,931528533	3,48062E-08	3,19725E-05
HES4	NM_021170	113,429251	1,928186694	3,60334E-08	3,19725E-05
PYHIN1	NM_198928	351,8613921	1,343790001	4,36155E-08	3,51818E-05
PYHIN1	NM_152501	351,8613921	1,343790001	4,36155E-08	3,51818E-05
PLXDC1	NM_020405	1249,201141	-1,598356793	8,56066E-08	6,60511E-05
RAB15	NM_198686	335,3830846	-1,227541695	1,0371E-07	7,37388E-05
SCRN1	NM_001145515	107,6206243	-1,794152408	1,03881E-07	7,37388E-05
TTC28	NM_001145418	215,0229678	-1,47211471	1,08174E-07	7,38329E-05
ENO1	NM_001428	5051,771479	1,071826567	1,70381E-07	0,000111985
HDGFRP3	NM_016073	162,2623951	1,523973369	2,62199E-07	0,000150096

STMN1	NM_203401	301,3859475	-1,252294653	2,60945E-07	0,000150096
STMN1	NM_005563	301,3859475	-1,252294653	2,60945E-07	0,000150096
STMN1	NM_203399	301,3859475	-1,252294653	2,60945E-07	0,000150096
SLC40A1	NM_014585	1234,282631	1,554877606	2,86228E-07	0,000158731
TRIM32	NM_001099679	151,1638134	-1,462662463	3,59059E-07	0,000187408
TRIM32	NM_012210	151,1638134	-1,462662463	3,59059E-07	0,000187408
EDARADD	NM_080738	926,1020547	0,967357565	4,41268E-07	0,000211641
NPAS2	NM_002518	327,4822981	1,399747863	4,38705E-07	0,000211641
EDARADD	NM_145861	926,1020547	0,967357565	4,41268E-07	0,000211641
GGT1	NM_001032364	173,1095945	1,396740036	4,7316E-07	0,000217893
TIMP2	NM_003255	170,749728	-1,582195639	4,78859E-07	0,000217893
GGT1	NM_013430	174,9784457	1,385499409	5,84562E-07	0,000230525
FLNB	NM_001164317	2588,109475	-0,977002483	5,47648E-07	0,000230525
FLNB	NM_001164318	2587,340095	-0,976336082	5,5911E-07	0,000230525
SNHG3	NR_002909	1319,250927	1,1382479	5,80958E-07	0,000230525
FLNB	NM_001457	2588,109475	-0,977002483	5,47648E-07	0,000230525
FLNB	NM_001164319	2587,340095	-0,976336082	5,5911E-07	0,000230525
GGT1	NM_001032365	173,9472264	1,382079891	6,51955E-07	0,000241033
GZMK	NM_002104	92,22733171	1,632718618	6,39043E-07	0,000241033
GGT1	NM_005265	173,9472264	1,382079891	6,51955E-07	0,000241033
MT2A	NM_005953	462,9884953	1,599130697	7,23163E-07	0,000261903
TCF12	NM_207036	941,565647	-0,932246606	7,93308E-07	0,000265624
TCF12	NM_003205	941,565647	-0,932246606	7,93308E-07	0,000265624
TCF12	NM_207038	941,565647	-0,932246606	7,93308E-07	0,000265624
TCF12	NM_207037	941,565647	-0,932246606	7,93308E-07	0,000265624
FLJ40330	NR_015424	142,0931259	-1,710444569	8,61281E-07	0,000281208
STAT4	NM_003151	1101,766992	1,21969605	8,71544E-07	0,000281208
NIN	NM_182946	1566,693386	-0,800818019	8,93085E-07	0,000283012
CYB561	NM_001915	99,12713612	1,630270478	1,00554E-06	0,000302446
CYB561	NM_001017917	99,12713612	1,630270478	1,00554E-06	0,000302446
CYB561	NM_001017916	99,23554395	1,630761861	1,00291E-06	0,000302446
ZC3H12D	NM_207360	486,282156	1,519531165	1,13055E-06	0,000334379
NIN	NM_016350	1555,669633	-0,795578742	1,20456E-06	0,000350428
LSR	NM_205835	323,9206118	1,536895304	1,23148E-06	0,000352481
TCF12	NM_207040	803,195413	-0,916029342	1,29194E-06	0,000363916
LSR	NM_205834	334,3300036	1,538269925	1,41593E-06	0,000388537
LSR	NM_015925	332,2247869	1,536484756	1,42313E-06	0,000388537
SNHG5	NR_003038	564,1615501	1,359682498	1,57707E-06	0,00042404
ADAMTSL5	NM_213604	97,68699663	1,604673919	1,65242E-06	0,000437669
PYHIN1	NM_198930	305,1711624	1,184134637	1,88629E-06	0,000485132
PYHIN1	NM_198929	305,1711624	1,184134637	1,88629E-06	0,000485132
FXYD5	NR_028406	6646,067492	1,105075045	1,99153E-06	0,000504881
FXYD5	NM_001164605	6627,79209	1,105738795	2,04134E-06	0,000506879
SCRN1	NM_001145514	113,8348128	-1,631807087	2,05653E-06	0,000506879
FXYD5	NM_014164	6625,615957	1,104915841	2,11089E-06	0,000507271

FXYD5	NM_144779	6626,511216	1,104695475	2,1153E-06	0,000507271
IGF2R	NM_000876	1946,143123	-0,955610797	2,33385E-06	0,000537878
SCRN1	NM_014766	113,5598049	-1,625417417	2,29672E-06	0,000537878
SCRN1	NM_001145513	113,4605218	-1,624754558	2,31514E-06	0,000537878
IFITM1	NM_003641	6231,804999	1,117478008	2,94191E-06	0,000669323
FAM195A	NM_138418	181,3649494	1,332898942	3,37359E-06	0,000757818
NIN	NM_182944	1547,828983	-0,801150328	3,58213E-06	0,000794606
LETMD1	NM_001024668	938,1264215	0,820807681	3,98676E-06	0,000873445
LETMD1	NM_015416	944,7864072	0,819123292	4,27019E-06	0,000924131
AOAH	NM_001637	314,3436279	-1,513958692	4,32227E-06	0,000924132
CAPN2	NM_001748	1531,572198	1,009388871	4,52588E-06	0,000956146
CAPN2	NM_001146068	1484,873489	1,011027218	4,97574E-06	0,001038817
SH3PXD2A	NM_014631	654,4324323	-0,959683476	5,24195E-06	0,00108167
SERTAD2	NM_014755	979,6436778	-0,760174029	5,79765E-06	0,001182587
DYNLT1	NM_006519	368,5529327	1,240590231	6,01473E-06	0,001212925
APP	NM_001136129	683,3827412	-0,795983948	7,65354E-06	0,001526065
GRIP1	NM_021150	145,7252269	1,259345555	8,0021E-06	0,001577836
HERC1	NM_003922	3927,834142	-0,804190618	8,62094E-06	0,001681179
OBSCN	NM_052843	3541,649059	-1,116803006	8,9033E-06	0,00171737
SLFN13	NM_144682	461,0640531	0,96390521	9,1636E-06	0,001748572
CCDC59	NM_014167	439,046112	1,217445347	9,66053E-06	0,001823784
SOCS2	NM_003877	457,6408321	1,417880811	1,02186E-05	0,001908826
CA6	NM_001215	202,4438639	1,40325237	1,23101E-05	0,002275567
TOB1	NM_005749	1401,989616	0,820122329	1,29345E-05	0,002366352
APP	NM_201414	714,1625049	-0,778417139	1,47894E-05	0,002678097
GSTK1	NM_001143681	2582,202001	0,788351954	1,53021E-05	0,002715516
TMPRSS3	NR_027348	52,25599357	1,524784671	1,5221E-05	0,002715516
MRC2	NM_006039	94,08695214	-1,372471155	1,90555E-05	0,003348106
EPHB6	NM_004445	1468,157264	-1,059512446	1,96894E-05	0,003425573
ACCN2	NM_020039	95,12254182	-1,518036124	2,03631E-05	0,003441553
ACCN2	NM_001095	95,12254182	-1,518036124	2,03631E-05	0,003441553
RNF157	NM_052916	2131,397587	0,997053057	2,00869E-05	0,003441553
TET1	NM_030625	129,6649284	-1,325266961	2,17071E-05	0,003634098
APP	NM_001136130	737,9595181	-0,743367394	2,30355E-05	0,003820442
AK1	NM_000476	608,1892416	1,015770196	2,44242E-05	0,004013259
AKAP11	NM_016248	1380,793733	-0,975152209	2,47539E-05	0,004030117
TBK1	NM_013254	89,06053306	1,359423494	2,57832E-05	0,004159535
FAM113B	NM_138371	4117,515264	0,841322789	2,88917E-05	0,004619033
GAPDH	NM_002046	5945,556307	0,978083478	2,9891E-05	0,004694204
HIGD2A	NM_138820	886,1170667	1,030391697	2,97574E-05	0,004694204
C8orf59	NM_001099673	304,3505756	1,055225693	3,23065E-05	0,00502904
GZMA	NM_006144	132,4012161	1,442415838	3,57034E-05	0,005415326
GALNT6	NM_007210	172,5735932	-1,386896996	3,55162E-05	0,005415326
C8orf59	NM_001099672	306,013797	1,057885535	3,5128E-05	0,005415326
CAMSAP1L1	NM_203459	201,3519058	-1,251390931	3,74657E-05	0,005634457

LY86	NM_004271	61,255401	1,428773163	4,05315E-05	0,005655278
C5orf13	NM_001142480	176,7311366	-1,312641332	4,33403E-05	0,005655278
C5orf13	NM_001142478	176,2065692	-1,314515026	4,245E-05	0,005655278
ANKRD50	NM_020337	34,98261422	-1,407672105	3,90519E-05	0,005655278
C5orf13	NM_001142481	176,2065692	-1,314515026	4,245E-05	0,005655278
C5orf13	NM_001142475	176,2065692	-1,314515026	4,245E-05	0,005655278
GSTK1	NM_001143680	3291,852661	0,784176828	4,33091E-05	0,005655278
TYMP	NM_001953	281,3841017	1,461539336	3,88121E-05	0,005655278
C5orf13	NM_001142477	176,2065692	-1,314515026	4,245E-05	0,005655278
C5orf13	NM_004772	176,2065692	-1,314515026	4,245E-05	0,005655278
C8orf59	NM_001099671	306,4415846	1,038669105	4,2249E-05	0,005655278
TYMP	NM_001113755	281,7093252	1,461911452	3,86509E-05	0,005655278
C5orf13	NM_001142474	176,2065692	-1,314515026	4,245E-05	0,005655278
FOSL2	NM_005253	261,772875	1,421289692	4,20691E-05	0,005655278
C5orf13	NM_001142479	176,7311366	-1,312641332	4,33403E-05	0,005655278
C5orf13	NM_001142476	176,2065692	-1,314515026	4,245E-05	0,005655278
C5orf13	NM_001142483	176,7056239	-1,317977683	4,07552E-05	0,005655278
C5orf13	NM_001142482	176,2065692	-1,314515026	4,245E-05	0,005655278
C8orf59	NM_001099670	307,9800423	1,043206872	4,47794E-05	0,005741563
SLC35F2	NM_017515	105,9821363	1,263906857	4,49722E-05	0,005741563
TYMP	NM_001113756	283,4368711	1,448844332	4,45817E-05	0,005741563
APP	NM_000484	768,7392818	-0,728365819	4,58782E-05	0,005815386
ETS1	NM_005238	27775,06212	-0,583754491	4,84558E-05	0,006098562
APP	NM_201413	767,5745402	-0,725464777	4,96493E-05	0,006204763
ZBTB34	NM_001099270	192,23974	-1,041642058	5,12508E-05	0,006338
RELB	NM_006509	434,8956767	1,161217185	5,14297E-05	0,006338
NHEDC2	NM_178833	171,2683093	1,08636314	5,23906E-05	0,006411886
PSME2	NM_002818	540,2612404	0,955208833	5,31564E-05	0,006422692
GSTK1	NM_015917	3364,610684	0,783559833	5,32027E-05	0,006422692
SNHG1	NR_003098	1866,221844	0,905086578	5,35931E-05	0,006426108
SUSD4	NM_001037175	154,4842007	1,33499541	5,84287E-05	0,006958896
ETS1	NM_001143820	26409,95751	-0,567351582	5,8841E-05	0,006961288
IRS1	NM_005544	227,5084219	-1,00432929	5,96524E-05	0,007010538
RCN3	NM_020650	136,5092201	-1,37526353	6,21042E-05	0,007250662
SARDH	NM_001134707	290,4460156	-1,107212797	6,44734E-05	0,007429508
SARDH	NM_007101	290,4460156	-1,107212797	6,44734E-05	0,007429508
VGLL4	NM_001128219	639,1082767	-0,965602542	6,90658E-05	0,007907367
ACTN1	NM_001130005	3536,091433	-0,752145737	6,98053E-05	0,007940797
GSTK1	NM_001143679	3587,043341	0,764657179	7,33883E-05	0,008242708
HMBOX1	NM_024567	244,4785586	-1,247524487	7,3357E-05	0,008242708
ETS1	NM_001162422	23372,23311	-0,549135151	7,51918E-05	0,008392162
IFITM2	NM_006435	2359,027335	0,886594183	7,59871E-05	0,008427924
YARS	NM_003680	1214,028189	0,85585763	7,67056E-05	0,008454767
S100A4	NM_019554	658,7557339	1,17847194	7,96106E-05	0,008562241
PASK	NM_015148	2370,564126	1,089597477	7,89663E-05	0,008562241

S100A4	NM_002961	658,7557339	1,17847194	7,96106E-05	0,008562241
ACTN1	NM_001130004	3613,203788	-0,747898057	7,94999E-05	0,008562241
CERKL	NM_001160277	1017,664539	0,912143562	8,36694E-05	0,008632546
CERKL	NM_001030311	1017,664539	0,912143562	8,36694E-05	0,008632546
CERKL	NR_027690	1017,664539	0,912143562	8,36694E-05	0,008632546
CERKL	NM_001030313	1017,664539	0,912143562	8,36694E-05	0,008632546
CERKL	NM_001030312	1017,664539	0,912143562	8,36694E-05	0,008632546
CERKL	NM_201548	1017,664539	0,912143562	8,36694E-05	0,008632546
CERKL	NR_027689	1017,664539	0,912143562	8,36694E-05	0,008632546
LDHA	NM_001135239	1614,585576	0,796142351	8,59064E-05	0,008812115
REST	NM_005612	473,2507283	-0,907261937	9,11163E-05	0,009292814
ACTN1	NM_001102	3578,015752	-0,738734255	9,27091E-05	0,00940123
RPLP1	NM_213725	7774,963237	0,985185238	9,328E-05	0,009405378
OXA1L	NM_005015	2155,64519	0,760547357	9,42157E-05	0,009446055
RPLP1	NM_001003	7923,008704	0,9841819	9,53683E-05	0,009507895
ACVR1	NM_001111067	347,0646855	0,876424601	9,6995E-05	0,009562625
ACVR1	NM_001105	347,2185614	0,874867072	9,67864E-05	0,009562625
SLU7	NM_006425	866,5328479	0,784276544	9,8286E-05	0,009614101
LDHA	NR_028500	1729,858762	0,765631535	9,86006E-05	0,009614101
OBSCN	NM_001098623	3814,562638	-1,016552209	0,000102199	0,009910531
SH3BGRL3	NM_031286	3405,481395	0,791991013	0,000109702	0,010580267
BCOR	NM_001123383	725,3775247	-0,987696527	0,000118844	0,011400031
SRGN	NM_002727	937,645626	1,079316389	0,000120461	0,011469259
BCOR	NM_001123384	724,915897	-0,986512131	0,000120858	0,011469259
MTHFD2	NR_027405	164,969752	1,246454832	0,000122133	0,011528557
HMBOX1	NM_001135726	249,5751949	-1,218933109	0,000123086	0,011557033
BCOR	NM_001123385	738,1414855	-0,990444542	0,000127723	0,011929308
BCOR	NM_017745	727,8136831	-0,982290979	0,000130389	0,012114551
MTHFD2	NM_006636	164,7190824	1,241398965	0,00013361	0,012349224
RGS12	NM_002926	182,3793082	-1,159654263	0,000134982	0,012411329
NUAK2	NM_030952	837,8370942	-0,946369267	0,000137299	0,012559313
EIF2C3	NM_024852	271,3143058	-1,073484327	0,000138864	0,012637298
MDGA1	NM_153487	236,9166367	1,335174508	0,000141403	0,012802774
NFKBIZ	NM_031419	2415,095712	0,85146429	0,000145598	0,013115675
IL16	NM_004513	7277,902554	-0,69493942	0,000149672	0,013280414
RAPGEF6	NM_001164390	607,0763698	-0,814875054	0,000149277	0,013280414
CHMP7	NM_152272	5496,188703	-0,602406198	0,00014857	0,013280414
SLCO3A1	NM_001145044	234,3899265	1,000237532	0,000151929	0,013413597
IL16	NM_172217	7285,833564	-0,692793012	0,000153163	0,013455626
FKBP11	NM_016594	279,9016362	1,135682948	0,000158496	0,01385554
NFKBIZ	NM_001005474	2387,185018	0,849965536	0,000162988	0,014040724
MDFIC	NM_001166345	1375,411607	0,746295185	0,000162669	0,014040724
MDFIC	NM_199072	1375,411607	0,746295185	0,000162669	0,014040724
STXBP1	NM_001032221	101,1206868	1,335909085	0,000167776	0,014186085
STXBP1	NM_003165	101,1206868	1,335909085	0,000167776	0,014186085

LDHA	NM_001165414	1814,91437	0,737276246	0,000165487	0,014186085
ISG15	NM_005101	710,3074121	1,1596122	0,000167873	0,014186085
LDHA	NM_005566	1814,517023	0,735842838	0,000170702	0,014356807
CHD9	NM_025134	456,2863782	-0,910697989	0,000174715	0,014624943
CBL	NM_005188	2328,795306	-0,77031814	0,000176138	0,014674886
RNF41	NM_005785	312,2634662	-0,830658288	0,000179012	0,014721161
RNF41	NM_194358	316,2693438	-0,819035481	0,000178554	0,014721161
ODC1	NM_002539	1242,87756	0,968799774	0,000179182	0,014721161
FLJ43663	NR_024153	336,7815284	1,029603068	0,000185046	0,015098287
B2M	NM_004048	60639,43833	0,621281479	0,000185474	0,015098287
RNF41	NM_194359	311,4791033	-0,825589817	0,000189381	0,015345882
LGALS9	NM_002308	731,1947503	1,058056563	0,000194308	0,015673551
LDHA	NM_001165416	1671,144308	0,736037298	0,000196564	0,015712734
C17orf49	NM_174893	928,8349423	0,903703368	0,000196056	0,015712734
ZC3H12A	NM_025079	761,107114	1,135718775	0,000197801	0,015740706
IL21R	NM_181078	802,2959279	-0,838468954	0,000200758	0,015758425
BCL9	NM_004326	549,1401067	-0,86758132	0,000199754	0,015758425
S100A10	NM_002966	740,2960277	1,035583278	0,000201447	0,015758425
C17orf49	NM_001142798	929,2965699	0,9015562	0,000201576	0,015758425
MARCKSL1	NM_023009	1218,402742	-0,653569161	0,000202559	0,015765864
NR3C1	NM_001020825	685,7008301	-0,672160828	0,000209871	0,016263629
RWDD1	NM_001007464	331,2314132	0,899320005	0,00021632	0,01661823
TGFBR2	NM_003242	4863,139184	-0,678776107	0,000215693	0,01661823
CD40LG	NM_000074	908,7654017	-0,751788884	0,00021818	0,016688906
RWDD1	NM_015952	312,8984504	0,922080467	0,000219735	0,016735718
LGALS9	NR_024043	720,3387643	1,050016113	0,000221976	0,016834115
OASL	NM_198213	70,96066065	1,310731718	0,000225272	0,016948839
SNX6	NM_152233	1046,817395	0,621314963	0,000226354	0,016948839
SNX6	NM_021249	1046,817395	0,621314963	0,000226354	0,016948839
UPP1	NM_003364	830,0156851	0,925458428	0,000232263	0,0173182
CREM	NM_182723	128,1976831	1,283778917	0,000239886	0,017437359
NBL1	NM_182744	128,1969642	1,307551924	0,000241722	0,017437359
CREM	NM_182722	128,1976831	1,283778917	0,000239886	0,017437359
NBL1	NM_005380	128,1969642	1,307551924	0,000241722	0,017437359
CREM	NM_182721	128,1976831	1,283778917	0,000239886	0,017437359
CREM	NM_182717	127,2170317	1,285665094	0,000237969	0,017437359
CREM	NM_182720	127,2170317	1,285665094	0,000237969	0,017437359
SBDS	NM_016038	1420,502374	1,047267676	0,000236993	0,017437359
RWDD1	NM_016104	339,176348	0,917190642	0,000246531	0,017712304
YBX1	NM_004559	2559,7143	0,7152804	0,000247748	0,017727943
CHST2	NM_004267	341,2077044	-1,122452657	0,000249994	0,017784214
TLR2	NM_003264	102,839121	1,201337284	0,000250538	0,017784214
SULT1B1	NM_014465	117,5514701	-1,273971252	0,000253867	0,017948731
FRY	NM_023037	277,9269909	-1,110080718	0,000256658	0,018074019
TJP3	NM_014428	118,8417186	1,289744393	0,000260431	0,018123959

NHP2	NM_017838	363,4150987	0,969951756	0,000259184	0,018123959
CLEC2B	NM_005127	859,2726539	0,903059071	0,000259915	0,018123959
FBXO11	NM_012167	360,1786609	-0,898026202	0,000262138	0,018148481
TGFBR2	NM_001024847	4900,982886	-0,675173573	0,000263851	0,018148481
BCL11A	NM_022893	73,08710532	-1,298604265	0,000263662	0,018148481
LGALS9	NM_009587	737,1500092	1,039715584	0,00026551	0,018192065
SYN1	NM_133499	37,23434696	1,248483762	0,000272028	0,018216636
SON	NM_138927	5734,871859	-0,644093328	0,000271153	0,018216636
LSM7	NM_016199	498,3209418	1,075743251	0,000270486	0,018216636
SYN1	NM_006950	37,23434696	1,248483762	0,000272028	0,018216636
NME2P1	NR_001577	1020,592831	0,831647638	0,00026798	0,018216636
FLJ43663	NR_015431	450,2550492	0,961984784	0,000267632	0,018216636
PDE7A	NM_002604	1498,352932	-0,717682518	0,000279203	0,018626859
KLF11	NM_003597	310,3425413	1,164327785	0,000281542	0,018642688
NCRNA00164	NR_027020	408,315051	1,172477321	0,000281331	0,018642688
UPP1	NM_181597	831,2488141	0,914969763	0,000284193	0,018748264
WHAMM	NM_001080435	1409,541815	0,982883176	0,000287868	0,018837313
APOL3	NR_027834	1434,273872	0,71112825	0,000288048	0,018837313
DEGS1	NM_144780	467,6233656	0,865823091	0,000288727	0,018837313
ATF4	NM_182810	3004,340726	0,738385555	0,000290536	0,018885931
APOL3	NR_027833	1452,130219	0,706988834	0,000291621	0,01888726
DCHS1	NM_003737	1151,628101	-1,085264534	0,000293666	0,018950544
MRPS21	NM_018997	595,9397414	0,794961385	0,000298498	0,019192556
VGLL4	NM_014667	603,5124751	-0,91238587	0,00030419	0,019487942
SON	NM_032195	5101,30353	-0,626747471	0,000307977	0,019659571
CREM	NM_182853	130,0135861	1,259266657	0,000309591	0,019691768
C7orf50	NM_001134396	305,3355452	1,010323803	0,000315526	0,019855768
C7orf50	NM_032350	305,3355452	1,010323803	0,000315526	0,019855768
C7orf50	NM_001134395	305,3355452	1,010323803	0,000315526	0,019855768
ZBP1	NM_030776	408,7584296	1,122148828	0,00031975	0,020006183
PRDX6	NM_004905	849,3462255	0,761145585	0,000321133	0,020006183
SIPA1L3	NM_015073	599,53547	-0,770997376	0,000321298	0,020006183
MRPS21	NM_031901	449,1726853	0,855464429	0,000326227	0,020224057
FOXO1	NM_002015	2794,691481	-0,624834066	0,000327077	0,020224057
RPLP0	NM_001002	21196,08406	0,834144636	0,000329638	0,020238899
ODZ1	NM_001163279	759,24552	-0,965438059	0,000333019	0,020238899
RPLP0	NM_053275	21201,51979	0,834137969	0,000330425	0,020238899
ODZ1	NM_001163278	759,24552	-0,965438059	0,000333019	0,020238899
ODZ1	NM_014253	759,24552	-0,965438059	0,000333019	0,020238899
LZTFL1	NM_020347	134,1132555	-1,067165818	0,000336945	0,020407617
ACVR2B	NM_001106	444,9766971	-1,100228371	0,000340381	0,020481835
ZNF696	NM_030895	116,6159699	-1,111443289	0,00034083	0,020481835
MYBL1	NM_001080416	97,9570667	1,121263532	0,000341633	0,020481835
CD97	NM_001025160	3815,866752	0,557996962	0,000347672	0,020773691
MYBL1	NM_001144755	96,65617284	1,111388761	0,000353238	0,020965109

IL21R	NM_021798	755,6386382	-0,812476021	0,000352091	0,020965109
OASL	NM_003733	88,45559002	1,272232804	0,000354609	0,0209763
STRN	NM_003162	340,0780341	-0,999518035	0,000369975	0,02178375
NFATC1	NM_172390	734,0562548	-0,747736693	0,000370714	0,02178375
CD97	NM_001784	3778,640462	0,558140262	0,000374003	0,021904502
DDB2	NM_000107	621,7708065	0,886031194	0,000377083	0,022012211
CCDC85B	NM_006848	630,7091289	0,905978707	0,000381877	0,022025482
EIF5A	NM_001970	2408,179625	0,88198602	0,000382275	0,022025482
EIF5A	NM_001143761	2406,102454	0,882468153	0,000378841	0,022025482
EIF5A	NM_001143760	2405,851784	0,882178178	0,000382192	0,022025482
ARHGAP26	NM_001135608	545,0039531	-0,682693963	0,000393259	0,022555506
ARHGAP26	NM_015071	546,0869155	-0,681149231	0,000395787	0,022555506
C8orf45	NM_173518	287,7828618	1,007730641	0,000396558	0,022555506
EIF5A	NM_001143762	2411,472493	0,880384902	0,000394083	0,022555506
TSC22D2	NM_014779	426,5585516	-0,738198318	0,000410112	0,023061758
APOL3	NM_145641	1356,474186	0,709188308	0,000411956	0,023061758
APOL3	NM_145642	1356,474186	0,709188308	0,000411956	0,023061758
EIF2C3	NM_177422	234,2523043	-1,044059254	0,000409299	0,023061758
APOL3	NM_145640	1356,474186	0,709188308	0,000411956	0,023061758
CD97	NM_078481	3832,710664	0,54764407	0,000418031	0,023328223
STMN1	NM_001145454	138,5209508	-1,108137405	0,000421272	0,023435407
SNRPG	NM_003096	163,5752802	1,029861933	0,000425091	0,023573946
HMBS	NM_000190	133,4778254	1,074336287	0,000437251	0,024172774
NME2	NM_001018139	1181,921348	0,832221074	0,000444354	0,024489126
ADAMTS10	NM_030957	780,8258932	-0,955011337	0,000448068	0,024617358
TNF	NM_000594	73,31993699	1,234063752	0,000458231	0,025020841
HPCAL4	NM_016257	254,9275934	1,039430321	0,000458076	0,025020841
APPL1	NM_012096	557,928552	-0,705702282	0,000469687	0,025226943
NME2	NM_001018138	1180,964625	0,8302039	0,000471757	0,025226943
NME2	NM_001018137	1180,964625	0,8302039	0,000471757	0,025226943
ABHD2	NM_152924	834,7634571	-0,669728032	0,000471957	0,025226943
PELI1	NM_020651	956,0634982	0,97194392	0,00046452	0,025226943
ABHD2	NM_007011	835,0712089	-0,670586298	0,000465197	0,025226943
NME2	NM_002512	1181,172316	0,830045372	0,000471622	0,025226943
ATF4	NM_001675	3200,729243	0,697042004	0,000479461	0,025422216
SLCO3A1	NM_013272	310,8478903	0,883693474	0,00048134	0,025422216
GPR132	NM_013345	493,5530022	0,940929464	0,000478987	0,025422216
SCAI	NM_173690	790,3116244	-0,888153327	0,000480779	0,025422216
APOL3	NR_027835	1402,094011	0,691643436	0,000494752	0,026053012
NRGN	NM_001126181	137,4514077	1,238913776	0,00050269	0,026102752
VIPR1	NM_004624	1525,304707	0,696783263	0,000503051	0,026102752
NRGN	NM_006176	137,4514077	1,238913776	0,00050269	0,026102752
PLSCR3	NM_020360	2629,136378	0,629762014	0,000497912	0,026102752
LOC100128731	NM_001134693	1425,154301	0,834181205	0,000500057	0,026102752
RARRES3	NM_004585	1042,847588	0,778481618	0,000505486	0,026137876

IL21R	NM_181079	763,9763643	-0,788422355	0,000506674	0,026137876
CTSW	NM_001335	404,6347723	1,087371087	0,000513202	0,026397927
EMP3	NM_001425	1561,378237	0,641558436	0,000521148	0,026510788
TTC16	NM_144965	288,0931874	1,098529367	0,000518333	0,026510788
SCAI	NM_001144877	786,1647842	-0,883070759	0,000520156	0,026510788
GADD45B	NM_015675	713,0845611	0,938168869	0,000521372	0,026510788
PLEKHG2	NM_022835	1287,733474	-0,768411262	0,000524575	0,026521687
ANKRD44	NM_153697	1201,939302	-0,770960181	0,000523831	0,026521687
LY6E	NM_002346	2608,046425	0,981563008	0,000530411	0,026664817
LY6E	NM_001127213	2608,046425	0,981563008	0,000530411	0,026664817
KLF13	NM_015995	6360,279601	-0,556697443	0,000534076	0,02677321
HMBS	NM_001024382	130,9786636	1,076653136	0,00054278	0,027132885
RGS12	NM_198229	154,6446129	-1,084945721	0,000546312	0,027232749
LDOC1L	NM_032287	231,3096551	-0,913624849	0,000550205	0,027347814
ZNF792	NM_175872	330,3403156	-0,854715144	0,000551703	0,027347814
FKBP11	NM_001143781	188,1689064	1,087583671	0,000579312	0,028636414
DRAP1	NM_006442	964,5697926	0,755538045	0,00059302	0,029232605
FAM13A	NM_014883	1427,570065	1,221281392	0,000601183	0,029552894
MAN1A2	NM_006699	535,0284147	-0,811931562	0,000605016	0,029659172
BAT2L	NM_013318	6677,583103	-0,533798103	0,000609149	0,029779507
NME1-NME2	NM_001018136	1282,107968	0,804772256	0,000616127	0,030037908
NDUFV2	NM_021074	309,8628253	0,816165163	0,000619416	0,030115476
FAM13A	NM_001015045	1438,058387	1,216955089	0,000629049	0,030500282
CD109	NM_133493	31,35566981	-1,13990647	0,000636853	0,030794509
GUK1	NM_001159390	1393,522185	0,825897905	0,000640876	0,030904832
C17orf49	NM_001142799	744,2769688	0,860183287	0,000643987	0,030970736
TAF4B	NM_005640	399,2476744	0,748947488	0,000653451	0,031340921
EPSTI1	NM_001002264	337,1993653	1,107595913	0,000658397	0,031408387
EPSTI1	NM_033255	337,1993653	1,107595913	0,000658397	0,031408387
ZNF394	NM_032164	1465,376075	0,932771469	0,000668463	0,031803072
DPP4	NM_001935	1579,604329	0,758609634	0,000672732	0,03192058
VGLL4	NM_001128221	568,0812329	-0,86036115	0,00068079	0,032216814
UQCRFS1	NM_006003	657,6242838	0,803870984	0,000685844	0,032369668
IDI1	NM_004508	529,0366119	0,930083098	0,000691793	0,032563797
GUK1	NM_001159391	1395,717995	0,819965561	0,000697035	0,032723762
TTC39C	NR_024232	177,0035455	1,008492476	0,000700598	0,032804258
NT5C3	NM_001002009	375,1954792	0,835812655	0,000715803	0,032823353
CBR3	NM_001236	40,59585636	1,198188544	0,00071094	0,032823353
MAN2C1	NM_006715	2622,252251	0,651107504	0,000704098	0,032823353
TP53TG1	NR_015381	140,6001582	1,135962134	0,000707342	0,032823353
CTDSP2	NM_005730	4956,890521	-0,511065082	0,000709236	0,032823353
SEL1L3	NM_015187	605,8326252	0,832692736	0,000714574	0,032823353
LBH	NM_030915	3939,52675	-0,856746181	0,000710962	0,032823353
NT5C3	NR_029372	375,1954792	0,835812655	0,000715803	0,032823353
GBP2	NM_004120	2334,748292	0,686421112	0,000718816	0,032876587

ZBP1	NM_001160417	387,6474742	1,069014498	0,000733613	0,033467069
CSTB	NM_000100	555,4480956	0,722576338	0,000736656	0,033519739
GTF3A	NM_002097	2121,211653	0,6520203	0,000739942	0,033583168
FTH1	NM_002032	5170,770567	0,921196928	0,000749438	0,033927376
B4GALT3	NM_003779	957,8107247	0,571251369	0,000751421	0,033930559
IFITM3	NM_021034	1878,952147	0,922111995	0,000768772	0,034546968
MAPKAPK3	NM_004635	888,1820169	0,693141312	0,000768965	0,034546968
NT5C3	NM_016489	373,7233975	0,833952443	0,000772257	0,034607253
SLC39A8	NM_001135148	273,9211566	0,77934088	0,000776176	0,034695245
H1FX	NM_006026	3189,234948	0,780426205	0,000784743	0,034990094
RAB25	NM_020387	81,06485687	1,18985279	0,000792117	0,035230326
CD109	NM_001159588	30,47506073	-1,11467013	0,000806051	0,03576044
MSH2	NM_000251	521,9995288	-0,873223219	0,000810566	0,035794988
NT5C3	NM_001166118	378,5749009	0,838105386	0,000810864	0,035794988
NR3C1	NM_001024094	1283,723151	-0,563412304	0,000825912	0,036189228
NR3C1	NM_000176	1283,723151	-0,563412304	0,000825912	0,036189228
CSDAP1	NR_027011	124,7626416	1,0880617	0,000822477	0,036189228
C19orf70	NM_205767	252,0464523	1,006241828	0,00082824	0,036201838
LTA	NM_001159740	325,7819878	0,884603844	0,000830775	0,036223407
VGLL4	NM_001128220	568,2685188	-0,851523243	0,000833959	0,036273142
FNBP1	NM_015033	3663,171514	-0,530575764	0,000845949	0,036615164
TNFRSF10B	NR_027140	826,6257348	0,696073184	0,000844154	0,036615164
PVT1	NR_003367	444,574841	0,838741695	0,000852469	0,036807596
TNFRSF1B	NM_001066	890,4045048	0,662064954	0,000854593	0,036809708
CLIC1	NM_001288	492,7564081	0,948831488	0,000864748	0,037156927
SYF2	NM_207170	615,8504957	0,785760242	0,000876846	0,037495221
ZBP1	NM_001160419	234,5956014	1,121801045	0,00087509	0,037495221
NR3C1	NM_001018076	1273,829336	-0,559460139	0,000883584	0,037692491
EMR4P	NR_024075	295,8747302	-1,054010972	0,000894574	0,037978744
PPA2	NM_006903	180,0658756	0,854944264	0,000893533	0,037978744
HINT1	NR_024610	1443,863456	0,771017014	0,000901929	0,038199597
NR3C1	NM_001018074	1272,526891	-0,557385831	0,000911881	0,038529163
RPL39	NM_001000	1248,561715	0,884433736	0,000916782	0,038552623
EPPK1	NM_031308	464,1445459	-1,083921387	0,000916529	0,038552623
NR3C1	NM_001018075	1272,402128	-0,557120535	0,000919167	0,038561544
MAP1LC3A	NM_181509	33,05510169	1,179556446	0,000923977	0,038619518
LDHA	NM_001165415	1534,4828	0,694531828	0,000924901	0,038619518
NR3C1	NM_001018077	1276,046224	-0,558374651	0,000931214	0,038791849
GPRC5B	NM_016235	168,8893982	1,154430998	0,000943399	0,039207385
LBXCOR1	NM_001031807	32,40230852	1,153264773	0,000949917	0,039268112
LOC388796	NR_015366	283,4898865	0,902924367	0,000951304	0,039268112
BTLA	NM_181780	177,7732134	-1,060716362	0,000951498	0,039268112
CREM	NM_183012	136,1916695	1,150016668	0,000955059	0,039323631
GUK1	NM_000858	1416,740853	0,791842947	0,000965633	0,039666942
CCNL1	NM_020307	3806,043732	0,631038251	0,000969533	0,039735161

OSGIN1	NM_013370	39,90208476	1,129089586	0,000975802	0,039756476
OSGIN1	NM_182980	39,90208476	1,129089586	0,000975802	0,039756476
PIK3CG	NM_002649	390,0435139	-0,90896572	0,000976774	0,039756476
IL17RA	NM_014339	1365,733895	-0,802797038	0,000980044	0,039765378
C10orf35	NM_145306	61,16907017	1,173950686	0,000981474	0,039765378
RAG1AP1	NM_001122839	375,5457759	0,895373088	0,000994908	0,040217855
TXNL1	NR_024546	502,2690702	0,64935429	0,00100317	0,040459652
TMPRSS3	NM_024022	56,43321987	1,166994464	0,001036824	0,041462573
ADCY9	NM_001116	167,9003048	0,893603497	0,001032484	0,041462573
TNFRSF10B	NM_147187	860,6633829	0,684529852	0,001041319	0,041462573
UHMK1	NM_175866	494,7367795	-0,696259424	0,001039433	0,041462573
PELI2	NM_021255	239,6461225	-0,892754853	0,001042055	0,041462573
CREM	NM_183011	140,6252512	1,123366741	0,00104166	0,041462573
NDUFA7	NM_005001	288,5639815	0,870049787	0,001053699	0,041832076
ARID1A	NM_139135	3489,147349	-0,622532117	0,001064899	0,042110376
ANKK1	NM_178510	42,62541212	1,156763364	0,001065455	0,042110376
RAB4B	NM_016154	520,2557628	0,693553054	0,00107375	0,042250057
GOLGA7B	NM_001010917	620,7347217	0,646706596	0,001073559	0,042250057
TXNL1	NM_004786	478,3506488	0,623409726	0,001086928	0,042579753
ACVR1C	NM_145259	96,86497923	1,102463073	0,0010869	0,042579753
NHP2	NM_001034833	262,1890872	1,007683534	0,001092449	0,042701755
SLC39A8	NM_022154	287,0913979	0,745097671	0,001117655	0,043590998
LOC388796	NR_027241	294,9068568	0,905772209	0,001122025	0,043665456
MAN2A1	NM_002372	921,7356462	-0,730079102	0,001127112	0,043767455
CDGAP	NM_020754	128,6751349	-1,016240035	0,001143619	0,044087525
BOLA2	NM_001031827	292,5013335	0,922776833	0,001145292	0,044087525
ZC3HAV1	NM_024625	2332,390213	-0,682771411	0,001143817	0,044087525
BOLA2B	NM_001039182	292,5013335	0,922776833	0,001145292	0,044087525
RPS29	NM_001032	4891,632265	0,739222163	0,001150986	0,044210818
P2RY11	NM_002566	1138,248351	0,687805322	0,001154889	0,04426494
SEMA4D	NM_001142287	2281,595983	-0,684336017	0,001166868	0,044614251
CREM	NM_183013	138,9882239	1,128082	0,001169031	0,044614251
KIAA1432	NM_001135920	365,764454	-0,750373765	0,001182978	0,045030689
ERC1	NR_027946	576,995997	-0,692065327	0,001185018	0,045030689
ARID1A	NM_006015	3858,379338	-0,6178251	0,0011999	0,045401773
RPL28	NM_000991	22931,56486	0,753603108	0,001197898	0,045401773
RPL28	NM_001136134	22939,6698	0,752980929	0,001209488	0,045667193
ZBTB44	NM_014155	1578,644569	-0,560770674	0,00122322	0,04608759
USP10	NM_005153	1052,388626	0,710197386	0,001237496	0,046526699
SYNE2	NM_182910	774,5629171	0,818733533	0,001244543	0,04669274
BCL3	NM_005178	354,5440348	1,037407442	0,001258702	0,046926331
LAMP3	NM_014398	209,1182523	1,146498569	0,001255564	0,046926331
RPL41	NM_001035267	560,307364	0,730904034	0,001256924	0,046926331
ZNF445	NM_181489	774,8494076	-0,881050497	0,00127452	0,047416418
RAG1AP1	NM_001122837	430,6746831	0,898379915	0,001278381	0,047460545

ODF3B	NM_001014440	71,93566304	1,106635229	0,001282431	0,04751151
HCST	NM_001007469	948,1195034	0,749409355	0,001298316	0,047800639
BTLA	NM_001085357	159,3534868	-1,077210555	0,001297191	0,047800639
HCST	NM_014266	948,1195034	0,749409355	0,001298316	0,047800639
ACVR1C	NM_001111032	96,10612448	1,087165983	0,00131019	0,048137941
COX7A2	NR_029466	462,8377497	0,923868841	0,001320909	0,048342215
SSNA1	NM_003731	453,558072	0,790412205	0,001321198	0,048342215
TP53INP2	NM_021202	134,1046419	1,14221168	0,001332754	0,048664719
NFKBIA	NM_020529	3855,164477	0,933619509	0,001337214	0,048727323
ACVR1C	NM_001111031	95,99771666	1,084917479	0,001346419	0,048785901
LTA	NM_000595	332,3858934	0,85378445	0,001342216	0,048785901
RPL41	NM_021104	552,9397256	0,731869887	0,001347069	0,048785901
CUX1	NM_181552	268,5809783	-0,941957539	0,001355725	0,048999398
RAG1AP1	NM_018845	458,1213697	0,880169862	0,001369771	0,049406414
ERC1	NR_027948	588,0891693	-0,686622356	0,001390635	0,049554623
THOC4	NM_005782	407,9711719	0,906931487	0,0013852	0,049554623
OSGIN1	NM_182981	39,80280161	1,085418016	0,001383675	0,049554623
RABGGTB	NM_004582	586,7754402	0,632670703	0,00137681	0,049554623
ERC1	NM_178039	588,0891693	-0,686622356	0,001390635	0,049554623
MAP3K7IP2	NM_015093	4314,542157	0,616129153	0,001379985	0,049554623

Supplemental table 2. Differential gene expression between CD31+ and CD31- naïve CD4+ T cells of thymectomized children

	Refseq id	**Base Mean**	**log2FoldChange**	**p-value**	**p-value adjusted**
PECAM1	NM_000442	283,2315153	1,819516026	3,32149E-17	7,66832 E-13
MAF	NM_001031804	191,9047606	-1,228786924	7,9188E-09	9,14107 E-05
FAM129C	NM_173544	71,36105381	1,105786384	9,52509E-08	0,000733019
MAF	NM_005360	95,1271272	-0,963829333	8,05523E-06	0,046492799

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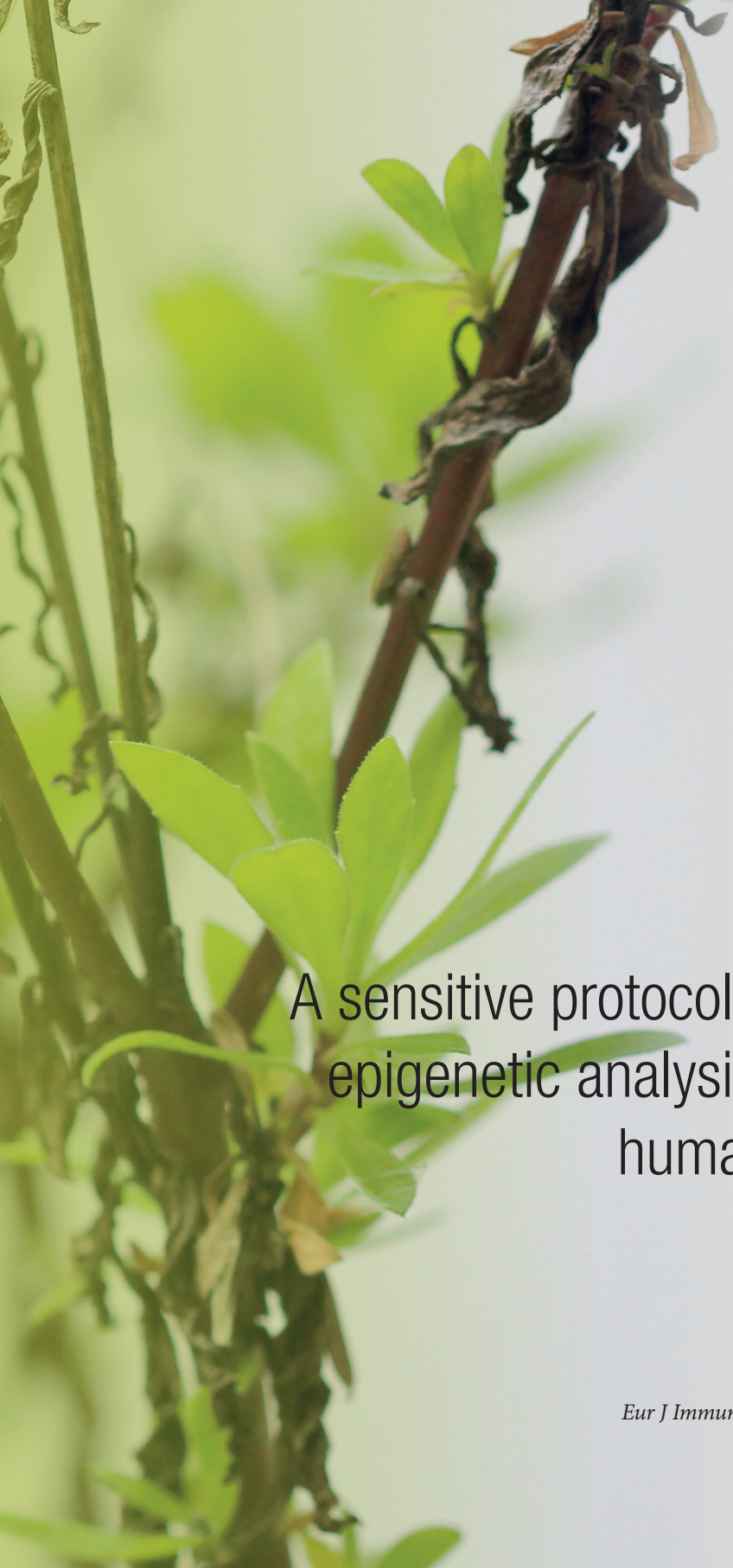

4

A sensitive protocol for *FOXP3* epigenetic analysis in scarce human samples

Eur J Immunol. 2014 Oct;44(10):3141-3

CD4+CD25high regulatory T (Treg) cells are key players in the maintenance of peripheral immune tolerance [1]. Stable expression of the FOXP3 transcription factor is essential for Treg cells' ability to suppress the immune responses of conventional T (Tconv) cells [2]. FOXP3 stability in murine Treg cells has been linked to *FOXP3* locus demethylation at the CNS2[3, 4], also called the Treg-specific demethylated region (TSDR) [5]. In contrast to FOXP3 expression[6, 7], complete demethylation of the TSDR [3], in addition to other genomic regions[8], is a true hallmark of human and mouse Treg cells, and its role in the early Treg lineage specification is currently under intense scrutiny in the mouse [3, 8, 9]. Unfortunately, the investigation of TSDR demethylation to define whether FOXP3+ T cells are bona fide Treg cells in translational research settings has so far been inaccessible due to limitations in clinical sample amount. Indeed, methods currently available to inspect the TSDR at single-CpG resolution [5] lack sensitivity due to bottlenecks at the amplification stage (Figure 1A). Other methods originally aimed at counting Treg cells in unsegregated populations [10] can be adapted to study the regulatory lineage commitment of sorted cells. However, because these methods separately amplify methylated and unmethylated TSDRs, the sample amount requirement is a function of the degree of TSDR methylation, growing exponentially at the two ends of the range, where one of the two species becomes limiting.

In this work, we report an inexpensive, single-CpG resolution, PCR-based protocol with very low requirements on sample amount and robust to aldehyde-based fixation, features making it of immediate relevance for sample-limited research settings. Our optimized protocol enhances the sensitivity of existing techniques [5] while retaining specificity. We introduced nested PCR, touchdown preamplification, primer tailing, and a two-step sequencing cycle (detailed protocol available as Supporting Information). The nested PCR targets 15 commonly investigated CpGs sites [5, 10], thereby allowing for direct comparison of results with previous literature. However, we found that measurements of the first CpG dinucleotide are typically noisier due to proximity to the sequencing primer, and we recommend excluding this first CpG dinucleotide, unless several technical replicates are performed. The touchdown preamplification step was introduced not to compromise specificity with the enhanced sensitivity. Moreover, we added 5' tails to the inner primers in order to (i) increase their length, which allows raising the annealing temperature, thereby minimizing spurious amplification; (ii) introduce C and G nucleotides, which are rarer in amplicons from bisulphite converted templates, thereby increasing DNA complexity and, consequently, the specificity of primer annealing; (iii) extend the amplicon at the 5' end, which allows to shift the sequencing primer upstream, thereby improving base resolution at the 5' end; and (iv) allow the sequencing primer to anneal only to the inner-PCR product. Finally, the two-step sequencing cycle yields a better signal balance when compared with the standard three-step denaturation/annealing/ extension cycle. Electropherograms were analyzed with ESME to quantitatively determine the methylated/unmethylated CpG ratio[11].

We favored Sanger sequencing for its widespread availability, but pyrosequencing is also an attractive option. Our method was able to successfully generate TSDR amplicons from much lower amounts of template than singleround PCR techniques (Figure 1A). The TSDR methylation profiles of CD25low/− Tconv cells and CD25highCD127low/− Treg cells generated by our protocol are in line with published data [5, 8–10], demonstrating its accuracy (i.e. proximity to the true value) in measuring highly methylated (Tconv cells), highly demethylated (male Treg cells), and partially demethylated (female Treg cells, due to *FOXP3* methylation on the inactive X chromosome) samples (Figure 1B). Accuracy was linearly preserved across the whole range of TSDR methylation, indicating no amplification bias of either methylated or unmethylated TSDR templates (Figure 1C). In addition, to carefully characterize the lower limit of detection, we calculated the percentage error (a normalized measure of the difference between observed and expected value, i.e. an estimate of accuracy) and the coefficient of variation (a normalized measure of data dispersion around the mean, i.e. an estimate of precision) of technical replicates across decreasing amounts of template gDNA. We were able to amplify down to 1.25 ng bisulphiteconverted gDNA with 100% success rates (Figure 1D). The percentage error remained below 5% for gDNA input down to 2.5 ng, and the coefficient of variation remained within 15% down to 5 ng (Figure 1E). Based on these data, we are confident that our optimized protocol allows a gDNA input as low as 5 ng. As few as 3 000 Treg cells were sufficient to obtain this amount of bisulphite-converted gDNA (Figure 1F). Below this threshold, stochastic sampling would degrade accuracy and precision. Finally, our protocol can be performed on cells that have previously undergone intracellular staining, as we obtained similar results when using either fresh (CD25highCD127low/−) or paraformaldehyde-fixed (CD25+FOXP3+) Treg cells (Figure 1G).

We showed the feasibility of our technique on Treg cells isolated from limiting samples of thymus and blood of thymectomized infants. Differently from circulating CD4+ T cells, a significant fraction of CD4+ thymocytes was positive for FOXP3 while remaining negative for CD25 (Figure 2A). As such, thymic samples were sorted as either total CD3+CD4+CD8−FOXP3+ or CD3+CD4+CD8−CD25+CD127−FOXP3+ cells. Our method was able to successfully measure the TSDR methylation profile of all sorted populations (Figure 2B), demonstrating its value for follow-up studies aimed at characterizing the human Treg lineage specification in the thymus.

In conclusion, we have optimized and validated an inexpensive, accessible, single-CpG resolution tool for *FOXP3* methylation analysis that is immediately applicable in translational research settings previously uncharted due to inaccessible requirements, which will help to shed new light on the development and stability of the Treg compartment in health and disease.

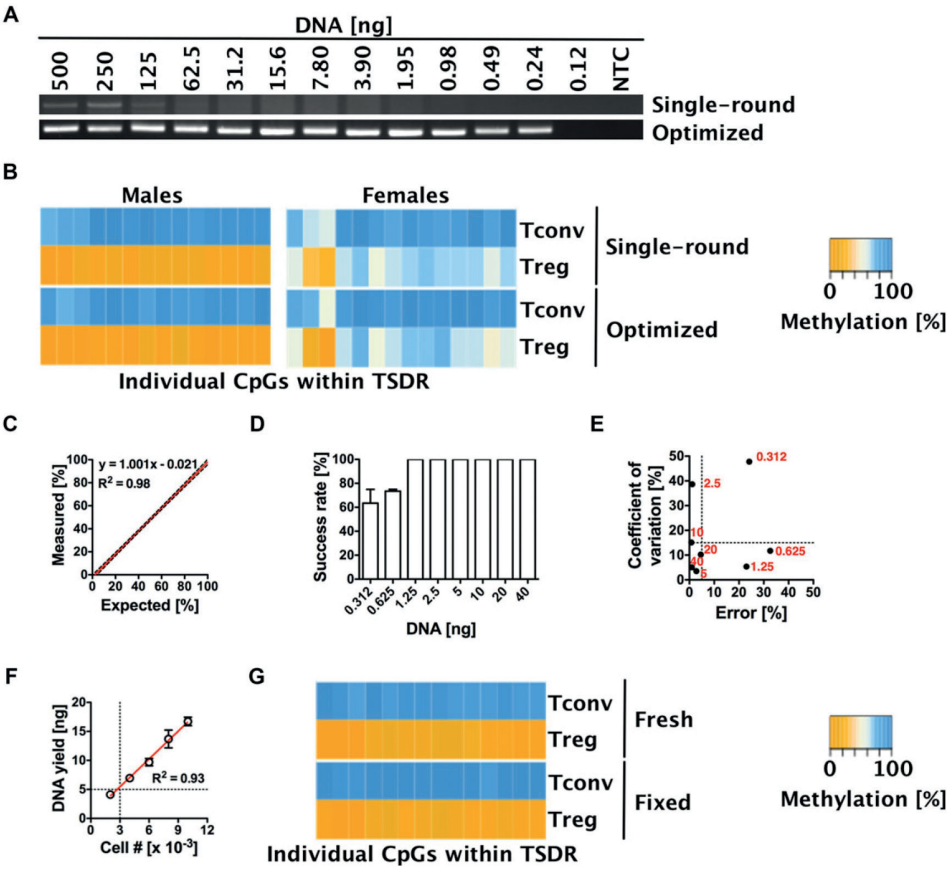

Figure 1. Technical characterization of the TSDR methylation analysis protocol. (A) Amplification performance of decreasing amounts of bisulphite-converted gDNA from CD4+ T cells using a single-round PCR protocol [5] or our optimized protocol. NTC, no template control. (B) Male and female Tconv cells and Treg cells were sorted as CD4+CD25low/– and CD4+CD25highCD127low/–. The degree of methylation for each CpG site was color-coded according to the legend. Two representative healthy donors out of eight analyzed are shown. (C) pUC57 plasmids carrying sequences corresponding to bisulfite-converted methylated and unmethylated TSDR were mixed at known ratios to generate increasingly methylated templates. Each reaction received 2500 plasmid copies. The methylation degree of four replicates per point was fitted to a linear model, which is displayed with 95% confidence intervals (gray area delimited by dotted lines). (D) The amplification success rate and (E) percentage error and coefficient of variation of four technical replicates amplified from male Tconv and Treg-cell samples mixed at 1:1 ratio are shown. Each point indicates the average of 14 CpGs, with the amount of bisulfite-converted gDNA per amplification (in ng) indicated in red. Dotted lines mark the thresholds of acceptance. (F) The yield of bisulphite-converted gDNA from varying cell numbers, FACS-sorted in lysis buffer, was fitted to a linear model. (G) Male Tconv cells and Treg cells were sorted as CD4+CD25low/– and CD4+CD25highCD127low/– (fresh), or CD4+CD25– and CD4+CD25+FOXP3+ (fixed). The degree of methylation for each CpG site was color-coded according to the legend. Data shown are representative of at least three independent experiments. In D and F, error bars represent the SD.

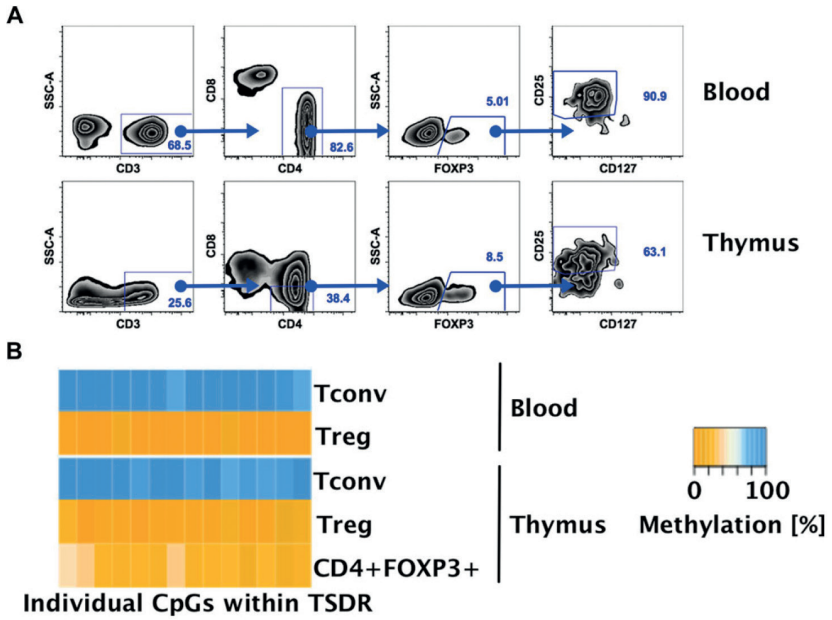

Figure 2. TSDR methylation of Treg cells isolated from pediatric samples. (A) CD25, CD127, and FOXP3 staining within CD3+CD4+CD8− T cells from peripheral blood and thymus. (B) Fixed cells from blood and thymus were sorted as CD3+CD4+CD8−CD25− (Tconv), CD3+CD4+CD8−CD25+CD127−FOXP3+ (Treg) or CD3+CD4+CD8−FOXP3+ (CD4+FOXP3+). The degree of methylation of each CpG site was color-coded according to the legend. Amplifications were performed using 15–20 ng bisulphite-converted gDNA, and two to three replicates were run per sample. A representative male infant out of five is shown throughout the figure.

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SUPPLEMENTAL INFORMATION

Samples

Adult blood was collected at the TSRI Healthy Blood Donor Service under SBMRI IRB approval upon written informed consent, and processed within 2 hours from withdrawal. Matched blood-thymus pairs from thymectomized infants undergoing heart surgery within their first 2 months of life were obtained at the Wilhelmina Children's Hospital. Neonatal samples were processed immediately after acquisition and FACS sorted the same day.

Flow cytometry and cell sorting

Treg cells (CD3+CD4+CD25highCD127low/- or CD3+CD4+CD8-CD25+CD127-FOXP3+) and Tconv cells (CD3+CD4+CD25low/- or CD3+CD4+CD8-CD25-FOXP3-) were sorted from either thawed adult PBMCs or neonatal fresh paired PBMCs and thymocytes using a FACSAria II (BD Biosciences). Intracellular staining was performed using the anti-human FOXP3 staining set from eBioscience, following manufacturer's instructions. Fluorochromeconjugated antibodies were from Biolegend, BD Biosciences and eBioscience. Analysis was performed with FlowJo (Treestar).

TSDR methylation

Large samples, for which DNA yields are not a concern, were processed using a two-step protocol: gDNA was first isolated with the ZR-Duet DNA/RNA MiniPrep kit (fresh cells) or the ZR FFPE DNA MiniPrep (fixed cells), then bisulphite conversion was performed with EZ DNA Methylation-Gold kit (all from Zymo Research). Smaller samples (103 to 105 cells) were processed using the single-step EZ DNA Methylation-Direct kit (Zymo Research) to avoid gDNA loss due to separate bisulphite conversion. Sorting cells directly into lysis buffer maximizes DNA yields. Digestion with the Proteinase K provided by the kit was performed for 20 min (fresh cells) or 4 hrs (fixed cells). The TSDR sequences of methylated and unmethylated TSDR were synthesized by AITbiotech and cloned into a pUC57 plasmid.

The TSDR of the FOXP3 locus was amplified by touchdown nested PCR using the Taq PCR core kit (Qiagen). The primary PCR was performed using the following primers: f: GTTTGTGGTTATTTTTGAAGT; r: CAAATAAACATCACCTACCAC. The secondary PCR was performed with modified Amp5 primers[5]: f: ACCAAC-TGTTTGGGGGTAGAGGATTT; r:AGTGGT-TATCACCCCACCTAAACCAA. The modified Amp5 primers have a 6-base tail at the 5' end (followed by a hyphen in the sequence reported above).

For the primary PCR, bisulfite-converted gDNA was amplified in a 20-ul reaction, using 1 U Taq enzyme, 1X buffer containing 1.5 mM MgCl2, 100 nM each primer, 0.2 mM dNTPs. Cycling conditions were as follows: 1) Initial denaturation: 94°C, 5 min; 2) Touchdown

amplification: 7 cycles of 94°C, 30sec; 58.5°C, 1 min (touchdown -0.5°C/cycle); 72°C, 30 sec; 3) Regular amplification: 25 cycles of 94°C, 30 sec; 55.5°C, 1 min; 72°C, 30 sec; 4) Final extension: 72°C, 5 min; 5) Hold: 4°C.

For the secondary PCR, 1 ul of 10-fold diluted primary PCR was amplified in a 20-ul final volume, using 1 U Taq enzyme, 1X buffer containing 1.5 mM MgCl2, 200 nM each primer, 0.2 mM dNTPs. Cycling conditions were as follows: 1) Initial denaturation: 94°C, 3 min; 2) Touchdown amplification: 5 cycles of 94°C, 30 sec; 60°C, 45 sec (touchdown -0.5°C/cycle); 72°C, 30 sec; 3) Preliminary amplification: 5 cycles of 94°C, 30 sec; 58°C, 45 sec; 72°C, 30sec; 4) Regular amplification specific to full-length tailed primers: 25 cycles of 94°C, 30 sec; 68°C, 30 sec; 72°C, 30 sec; 5) Final extension: 72°C, 5 min; 6)Hold: 4°C.

Successful amplification was confirmed by running 5 ul PCR products in a 2% agarose gel. PCR products were cleaned up enzymatically using ExoSAP-IT (Affymetrix) according to the manufacturer's instructions, then sequenced using the following HPLC-grade primer: ACCAACTGTTTGGGGGTA. The sequencing reaction was performed in 10 ul final volume using 5 ng cleaned PCR product, BigDye Terminator v3.1 (Life Technologies) at 1/8 strength in 1x buffer (1 ul BigDye, 1.5 ul 5x buffer), and 0.3 uM primer. Cycling conditions were as follows: 1) Initial denaturation: 96°C, 1 min; 2) Cycle sequencing: 25 cycles of 96°C, 10 sec; 50°C, 2 min; 3) Hold: 4°C. The reaction was cleaned up using the BigDye XTerminator kit (Life Technologies) according to the manufacturer's instructions, and then loaded on a Genetic Analyzer (Applied Biosystems). We observed that best results were obtained when the KB basecaller was used on traces generated by the 3100 Genetic Analyzer with the POP4 polymer, or when the SR basecaller was used on traces generated by the 3730 DNA Analyzer with the POP7 polymer. Electropherograms were analyzed with ESME v3.2.5[11], obtained from www.epigenome.org/index.php?page=download. ESME was executed on a 64-bit Linux Mint distribution, with the following packages installed: ia32-libs, lib32stdc++6, libc6-i386, lsb-core. A Linux script to consolidate ESME output from multiple trace files and the FOXP3 TSDR reference file are available upon request to the corresponding authors.

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5

Human neonatal thymectomy induces altered B cell auto-reactivity later in life

Submitted

Background
Human neonatal thymectomy results in a temporary decrease in (mainly naïve) T-cell numbers. Peripheral homeostatic proliferation (HP) of T-cells after lymphopenia has been associated with the development of autoimmune disease in experimental models. So far, it is unknown how removal of the thymus at young age affects the B-cell compartment and the development of auto-reactivity.

Objective
To investigate the effect of neonatal thymectomy on B-cell homeostasis and the development of auto-reactivity.

Methods
Children who had undergone cardiac surgery necessitating total neonatal thymectomy were compared to healthy age-matched controls. T- and B-cell phenotyping, antigen microarray and (auto)-antibody analyses were performed. A cohort of young (1-5 years post-thymectomy) and older children (>10years post-thymectomy) were evaluated to assess the effect of thymic regeneration on (persistence of) B cell auto-reactivity.

Results
Neonatal thymectomy revealed a different auto-antibody profile, but did not result in autoimmune disease. Following neonatal thymectomy, HP correlated with, and preceded detection of auto-antibodies; In the older cohort, over 60% of the individuals displayed auto-antibodies that did not correlate with T-cell numbers or thymic output suggesting that auto-reactivity persists despite thymic regeneration.

Conclusion
Neonatal thymectomy results in temporary T-cell lymphopenia and peripheral homeostatic proliferation. This T-cell expansion is associated with and precedes the development of auto-antibodies. Concomitant preferential expansion of regulatory T-cells during homeostatic proliferation might play a role in preventing auto-reactivity from becoming clinically overt autoimmune disease. Finally, our findings suggest that partial removal of the thymus in cardiac surgery patients may be preferred over total removal of the thymus.

INTRODUCTION

The thymus is the major production site of T cells, starting at around 12 weeks of gestation.[1] After the first years of life its function diminishes, but is maintained into adulthood although at a much lower level. [2] In adulthood T cell homeostasis is mainly provided by peripheral homeostatic proliferation (HP). [3] This is also apparent as no change in T cell number is seen after therapeutic thymectomy in adults with myasthenia graves. [4 5 6] However, the role of the thymus in T cell homeostasis might be more prominent very early in life; indeed, neonatal thymectomy has been shown to result in T cell lymphopenia and skewing towards a memory phenotype. [7, 8] In neonatal cardiac surgery the thymus, which obstructs access to the heart and great vessels, is routinely removed. Besides transient lymphopenia, these children show no clinical signs of immune deficiency or immune deregulation and are otherwise immunologically competent.[8, 9] Subsequent to neonatal thymectory, antibody titers to previous vaccinations are relatively unaltered.[10,11] However, responses to new vaccinations seem to be delayed or sometimes absent. [12, 13] This suggests that B cell homeostasis and the antibody repertoire may be affected by neonatal thymectomy. The thymus has a clear role in the development of the T cell repertoire, but also indirectly affects the B cell repertoire via T-cell help and suppression. Cohen and colleagues previously showed that the IgM autoantibody repertoire is shared by most newborns and is primarily directed to relatively uniform sets of self-antigens; the IgM autoantibody reactivities of the healthy mothers show a significantly lower correlation. [14, 15] This suggests that the natural autoantibody repertoire of humans begins with a standard set of autoreactive antibodies, which later diverge as a result of individual immune experience during life. While IgG auto-reactive antibodies are often associated with autoimmune disease, IgM auto-reactive antibodies have been associated with maintenance of self-tolerance.[16, 17] The role of the thymus in the developing antibody repertoire remains to be elucidated.

Thymopoiesis results in the generation of new polyclonal naïve T cells, and HP expands the existing peripheral T-cell pool. Homeostatic proliferation is driven by cytokines, predominantly interleukin-7 (IL-7), and in part by recognition of self-peptide/MHC ligands. [18, 19] Recovery from T cell lymphopenia, via homeostatic proliferation, may therefore result in expansion of auto-reactive T cells and could result in some degree of loss of self-tolerance and alterations in the autoantibody repertoire. An association between T cell lymphopenia and autoimmune disease is recognized, as several autoimmune diseases are associated with low T cell numbers. [20] Furthermore, in animal models lymphopenia is a factor that drives the development of autoimmunity. [21 22] It is currently unknown whether thymectomy at early age in an otherwise healthy person will result in increased auto-reactivity and whether such auto-reactivity necessarily leads to autoantibodies and autoimmune disease.

Here, we utilized a unique cohort of neonatally thymectomized children to assess the role of the thymus in B cell homeostasis and the development of the autoantibody repertoire. In addition, we assessed the role of thymic tissue regeneration, which has been shown to occur in the majority of children later in life. [8] Using this cohort, we now show that in the first years after neonatal thymectomy, homeostatic proliferation is increased and is associated with alterations in the autoantibody repertoire. Even though none of the individuals manifested with overt autoimmune disease the majority of post-thymectomy patients displayed enhanced levels of autoantibodies binding to nuclear antigens (ANA) and other self-molecules. This was apparent up to 29 years after neonatal thymectomy, despite thymic regeneration and restoration of general T cell levels. Together, these findings imply that, in terms of autoantibody repertoires, the immune system forgives but does not forget prior thymectomy and T cell lymphopenia.

METHODS

Patient selection and characterization

Patients who had undergone complete thymectomy during infancy because of surgery to treat congenital heart defect at the Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands, were included in this study. The age at which these patients were thymectomized was within the first month of life (8.4 +/-5.9 days, median +/-SD). Blood samples were taken during follow-up at 1-5years (n=10-25) and after approximately 10 years (n=26) of neonatal thymectomy, due to previously shown possibility of thymic tissue regrowth after 5-10 years.[8] Exclusion criteria were clinical signs of infection at time of blood draw and the presence of a syndrome or genetic disorder (e.g. 22q11 deletion, trisomy 21). Clinical reports of all patients were available and were screened for the presence or indication of autoimmune disease at the time of blooddraw.

A healthy control group, without major neonatal surgery, consisted of 1-5 year (n=10-31) and >10 year (n=11) old age-matched healthy children, who visited the University Medical Center Utrecht to undergo elective surgery and were considered immunologically healthy. The study was approved by the medical ethical committee of the University Medical Center Utrecht and written consent was obtained from all study participants or their legal guardians in agreement with Helsinki Declaration of 1975, revised in 1983. As cell counts and sufficient cells were not available for all samples due to the limitation in allowed blood amount taken from children, some data points are not shown for all study subjects.

Cell preparation and flow cytometry

PBMCs were isolated from heparinized blood samples by using the Ficoll Isopaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden), and viably

frozen and stored in liquid nitrogen until further processing. The flow cytometry staining protocol is described elsewhere.[23] Flow cytometry antibodies are described in the supplementary data.

Auto-antibody measurement

Plasma samples from thymectomized individuals were tested for autoantibodies at a 1:100 dilution or in the case of anti-neutrophil cytoplasmatic and perinuclear autoantibodies at a 1:10 dilution using a commercially available indirect immunofluorescence assay, performed according to the manufacturers protocol (Euroimmun, Lübeck, Germany). Other autoantibodies tested and their concentrations are described in supplementary data.

Antigen microarray

Antigen microarray chips were prepared as described elsewhere. [14, 24, 25] Briefly, 911 antigens, each at its optimal concentration, were spotted in tetraplicates on epoxy-activated glass substrates using a 48-pin robot (printed at ImmunArray Ltd, Israel, MicrogridII arrayer MG610, Genomics/Digilab). These antigens included proteins, synthetic peptides from the sequences of selected proteins, nucleotides, phospholipids, and other self and non-self molecules. The arrays were washed after blocking and incubated for 1 hr at 37°C with a 1:500 dilution of two detection antibodies, mixed together: a goat anti-human IgG Cy3-conjugated antibody, and a goat anti-human IgM Cy5-conjugated antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Image acquisition was performed by laser (Agilent Technologies, Santa Clara, CA) and the signal intensity were extracted using in house software. The quantitative range of signal intensity of binding to each antigen spot was 0.01–65 000; this range of detection made it possible to obtain reliable data at the 1:10 dilution of test samples.

Measurement of immunoglobulins and Free light chains

Total IgG and IgM concentrations were determined by nephelometry according to the manufacturers instruction (Beckman Coulter, Woerden, NL) using an Image 800 nephelometer. Total plasma FLC concentrations were determined using an ELISA adapted from Abe et al. [26, 27] In short, plates were coated with goat-anti mouse IgG (M4280, Sigma) and subsequently incubated with mouse-anti human kappa or lambda Ig-FLC monoclonal antibodies (obtained from Dr. A. Solomon, Tennessee). After incubation with different dilutions of samples and standards (The BindingSite), plates were incubated with HRP-labeled goat F(ab')2-anti human kappa or lambda Ig light chain Ab's (AHI1804 and AHI1904, respectively, Biosource, USA). Finally, TMB was used as a substrate and the reaction was terminated by adding 0.9M H2SO4. At least three data points within the linear portion of the standard curves were used to estimate the FLC concentration.

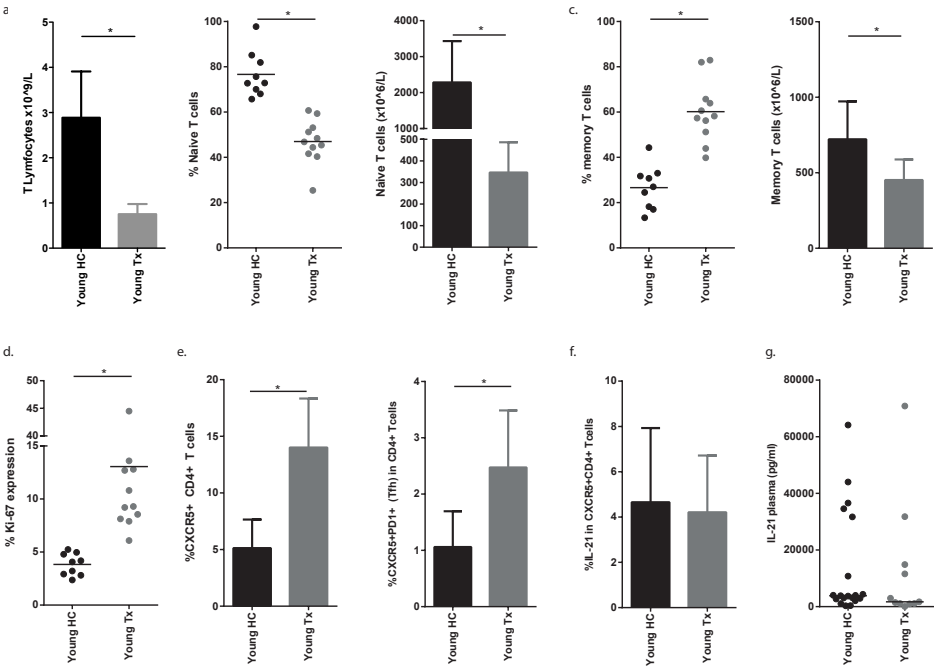

Figure 1. Neonatal thymectomy results in lymphopenia-induced T-cell proliferation
a)Lymphocyte count in healthy control (young HC, n=9) and thymectomized (young Tx, n=10) children **b)** Proportion and numbers of naïve CD3+ T cells in HC (n=9) and Tx (n=11) (left and right panel) **c)** Proportion and numbers of memory CD3+ T cells in HC (n=9) and Tx (n=11) (left and right panel) **d)** Percentage of proliferation (ki-67+) in CD3+ T cells of HC (n=9) and Tx (n=11) **e)** Percentage CXCR5+ in CD4+ T cells (left panel), follicular T helper cells (CXCR5+PD-1+) in CD4+ T cells in HC (n=9) and Tx (n=11) **f)** IL-21 expression in Tfh cells in HC (n=9) and Tx (n=11) **g)** IL-21 plasma concentration (pg/ml) of HC (n=19) and Tx (n=11), median shown. Mean (and SD) depicted in other panels. * for P<0.05

Luminex

IL-21 was measured in thawed plasma by multiplex technology (xMAP, Luminex, Austin, TX USA). The immunoassay was performed as described previously.[28] Aspecific heterophilic immunoglobulins were preabsorbed from all samples with heteroblock (Omega Biologicals, Bozeman MT, USA). Acquisition was performed with the Biorad FlexMAP3D (Biorad laboratories, Hercules USA) in combination with xPONENT software version 4.2 (Luminex). Data analysis was performed with Bioplex Manager 6.1.1 (Biorad).

Statistics

Statistical significance between two groups was assessed using the Mann-Whitney U test for unpaired data and Wilcoxon signed rank test for paired data. Statistical difference is indicated with * for P<0.05. Differentially reactive self-antigens were defined based on thresholds (p-value<0.05, FDR<0.2, T-test>abs(1.5)). Unsupervised hierarchical clustering

dendrogram analysis of the antigen microarray data was performed using sample distance metric of one minus pearson correlation. Further analysis of the antigen microarray assay is described in the supplementary data.

RESULTS

Neonatal thymectomy results in lower T cell numbers, increased T cell proliferation and an increase in follicular T helper cells

We first examined T cell lymphopenia in a cohort of children 1-5 years following neonatal thymectomy (young Tx). In this Tx group, T cell numbers were significantly decreased compared to healthy age-matched controls (young HC) (figure 1a). Within the T cell compartment, both the proportion and the absolute numbers of naïve T cells were significantly reduced (figure 1b, left and right panel respectively). Concurrently, there was an increase in the proportion of memory T cells (figure 1c, left panel) but a decrease in the absolute number of memory T cells (Figure 1c, right panel). Proliferation of T cells, as measured by the percentage of ki-67 expressing CD3+ T cells, was significantly increased in thymectomized children (figure 1d). Upon antigen activation, follicular T helper cells (Tfh) are necessary to help B cells generate specific antibodies. The proportions of CXCR5+ and CXCR5+PD1+Tfh cells were increased in thymectomized children (figure 1e, left and right panel respectively). No difference in IL-21 expression of Tfh cells or in IL-21 plasma levels between the Tx and HC group was detected (figure 1f and 1g respectively).

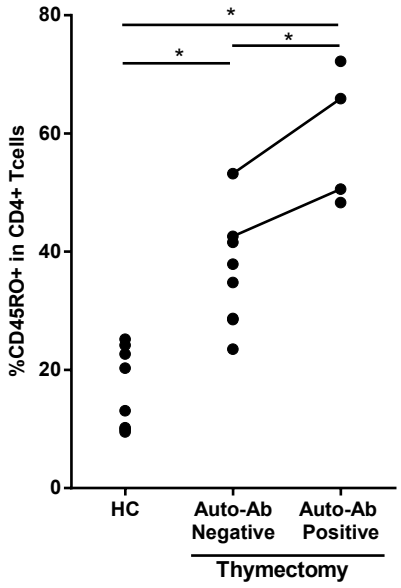

Figure 2. Memory T cell expansion precedes development of autoreactivity
Percentage memory CD4 T cells in HC (n=9), autoantibody negative (n=8) and positive (n=4) young Tx patients. (Line indicates same patient (patient 3 and 9), but at different time). * for P<0.05

Overall, neonatal thymectomy resulted in a decrease in T cell numbers and specifically naïve T cells, with a concomitant increase in proliferation and a skewing towards the memory compartment. In addition, thymectomized children had an increased proportion of follicular helper T cells.

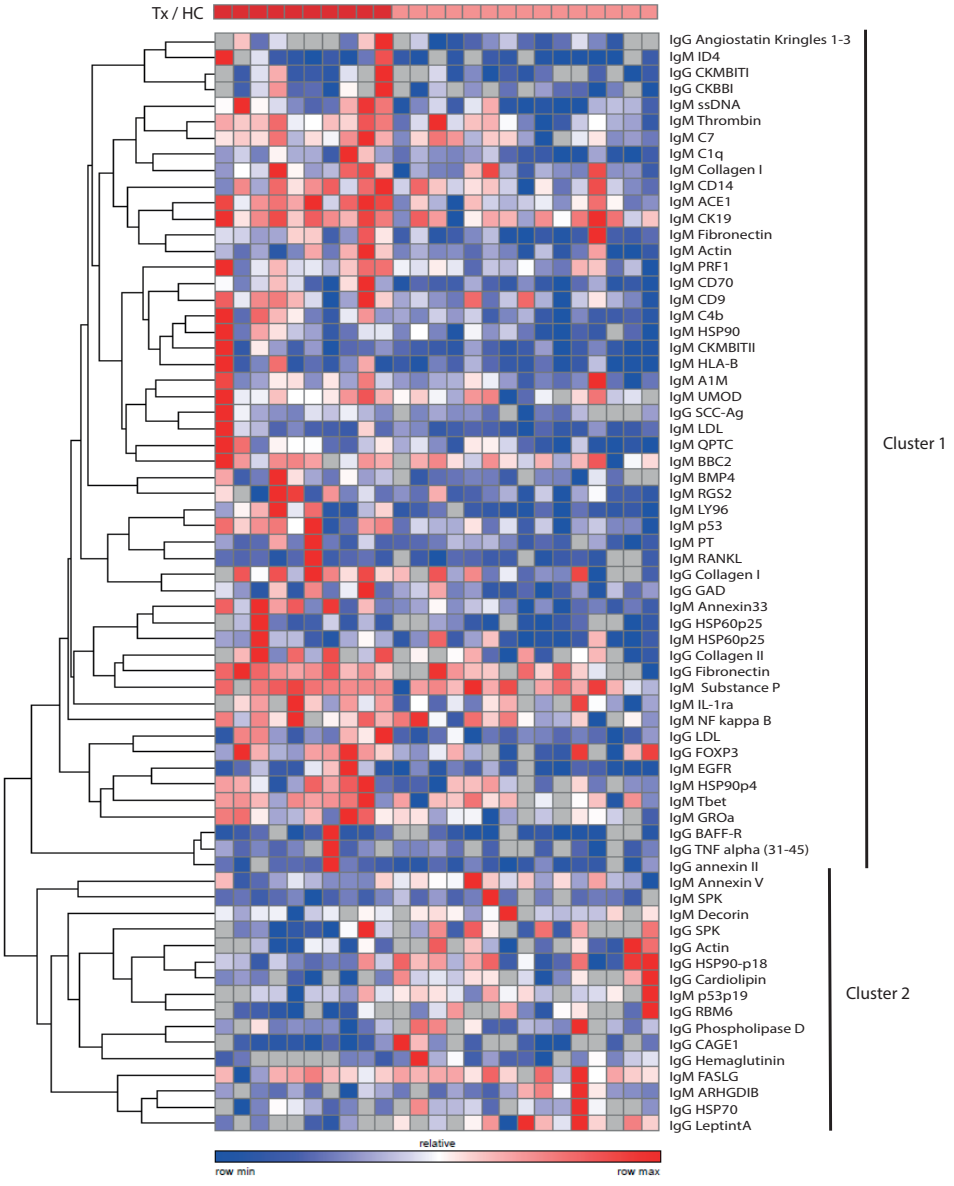

Figure 3. Thymectomized children manifest an altered autoantibody reactivity profile. IgM and IgG self-antibody profile analysis of 68 differentially expressed antigens of HC (n=10, red) and Tx (n=15, pink).

Table 1. Antigen reactivity of the autoantibodies differentially expressed between thymectomized and healthy children corrected for multiple comparison (False discovery rate (FDR)), data shown as 'mean (SD)'

Antigen Reactivity	Young HC	Young Tx	p-value
IgM ACE1	-0,02 (0,20)	-0,38 (0,19)	0.027
IgM LY96	1,67 (1,38)	-0,08 (0,37)	0.027
IgM Annexin33	0,21 (0,44)	-0,31 (0,22)	0.032
IgM Thrombin	-0,19 (0,12)	-0,42 (0,23)	0.032
IgM p53	0,16 (0,37)	-0,24 (0,21)	0.032
IgM DNAss	7,58 (3,67)	3,44 (2,62)	0.040
IgM CD70	0,56 (0,63)	-0,08 (0,27)	0.032
IgM C1q	-0,01 (0,27)	-0,28 (0,16)	0.027
IgM C4b	1,05 (0,93)	0,17 (0,43)	0.040
IgG LeptintA	-0,82 (0,06)	-0,59 (0,16)	0.027
IgG Cardiolipin	-0,78 (0,05)	-0,66 (0,08)	0.027

Development of ANA and ANCA autoantibodies after neonatal thymectomy correlates with high percentage of CD4 memory T cells

To assess the presence of autoantibodies after thymectomy, a selection of autoantibodies commonly used in clinical diagnostics was measured. In 4 out of 10 thymectomized patients, autoantibodies were detected against nuclear antigen (ANA, n=2, patient 8 and 9) and neutrophil cytoplasmic antibodies (ANCA, n=2, patient 3 and 7)(suppl. table 1). While ANA antibodies are also described in a minority of healthy children, this is not the case for ANCA antibodies as they are usually associated with autoimmunity. [29]

These four autoantibody positive children manifested a higher proportion of memory T cells compared to autoantibody-negative thymectomized children and healthy controls (figure 2). Interestingly, in two out of the four children a prior sample was available; 11 and 37 months before the detection of autoantibodies, a high proportion of memory T cells was already present in these children (patient 3 and 9, suppl. table 1), a time at which they were still autoantibody negative (connecting line, figure 2). This suggests that homeostatic proliferation after neonatal thymectomy may indeed induce B cell auto-reactivity.

Altered autoantibody repertoires following neonatal thymectomy

To assess the autoantibody profile after neonatal thymectomy, we studied sera using an antigen microarray containing 911 different antigens. Both IgM and IgG autoantibodies were determined in subjects 1-5 years post-thymectomy as well as healthy age-matched controls. A total of 68 autoantibodies were found to be altered in the thymectomized compared to the healthy children. Cluster analysis of the differential autoantibody reactivities between the two groups resulted in two autoantibody reactivity clusters (figure 3). Cluster 1 showed a lower autoantibody intensity in thymectomized children; these

decreased autoantibodies consisted mostly of IgM autoantibodies. In contrast, cluster 2 showed increased autoantibody intensity in thymectomized children; these increased autoantibodies were enriched for the IgG isotype. After adjustment for multiple testing; the intensity of several IgM autoantibodies were still significantly lower in thymectomized children (table 1) as follows: anti-ssDNA (single stranded DNA), anti-C1q, anti-C4b, anti-ACE1 (Angiotensin-converting enzyme 1), anti-LY96 (lymphocyte antigen 96), anti-Annexin 33Kda, anti-Thrombin, anti-p53, and anti-CD70. Concurrently, thymectomized children manifested increased IgG autoantibody intensities towards cardiolipin and leptintA (leptin triple antagonist) (table 1). IgG autoantibodies are often associated with overt autoimmune diseases, IgM autoantibodies, in contrast, are associated with prevention of autoimmune disease. [4, 30, 31] The clinical consequences of these repertoire alterations are not known at present.

Neonatal thymectomy does not affect B cell compartment phenotype

An altered autoantibody profile could be due to differences in B-cell reactivity, as seen in several autoimmune diseases (for instance; systemic lupus erythematosus and systemic sclerosis) [32, 33]. We therefore analyzed both B-cell differentiation and immunoglobulin production 1-5 years post-thymectomy. The proportion of $CD19^{+}$ B-cells from total lymphocytes did not significantly differ between thymectomized children and healthy children. Further analyses of the immature/transitional, mature naïve, memory and antibody-secreting stages of B cells showed no differences between the groups. $CD21^{-/low}CD19^{high}$ and $CD19^{+}CD5^{+}$ B cells have been associated with autoimmune disease, but also within these subsets no differences in percentages were found (table 2).

The level of total immunoglobulin and free light chains could shed further light on changes in B cell function. Total IgM-levels did not differ, but total IgG levels were significantly lower in the thymectomy group. No difference in the levels of kappa and lambda free light chains and their ratio were apparent between the groups (supplemental figure 1).

Overall, neonatal thymectomy did not result in an altered B cell compartment except for a decrease in total IgG levels.

Altered autoreactivity in thymectomized patients persists despite restoration of thymic output and T cell numbers

We wondered if altered autoantibody repertoires might persist or even further develop, later in life in the thymectomized subjects. Taking into account that thymic tissue regenerates later in life in the majority of thymectomized children [8], we examined ANA and ANCA autoantibodies in an older cohort of children with an average follow-up of 16 years after neonatal thymectomy (9.1-29 years range). In this older thymectomized group, T cell numbers were restored and CD4+ T-cell proliferation was similar to healthy controls (figure 4a, 4b). Remarkably, in the majority of older children (16 out of 26) autoantibodies

Table 2. Phenotypical analysis of B-cell subsets, mean (SD)

Phenotype	HC (n=7)	Tx (n=11)	p-value
CD19+ B-cell (within lymphocytes)	19.8 (10.2)	26.3 (5,6)	0.179
Immature/Transitional (IgD+CD27-):			
T1, CD38^{high} CD10+	2.6 (0.9)	2.3 (0.7)	1.000
T2, CD38+ CD10+	1.7 (0.2)	1.8 (0.1)	0.930
T3, CD38+ CD10-	13.6 (2.4)	15.6 (2.4)	0.211
Mature Naïve (IgD+CD27-):			
CD38- CD10-	60.2 (7.1)	58.1 (10.2)	0.285
Memory B-cell:			
IgD-CD27-: Double negative	5.4 (3.6)	5.5 (2.4)	0.596
IgD+CD27+: Non-switched	4.2 (2.0)	3.1 (1.4)	0.328
IgD-CD27+IgM+: IgM memory	3.8 (3.0)	3.1 (1.8)	1.000
IgD-CD27+IgM-: switched	1.0 (0.9)	0.9 (0.4)	0.536
Antibody secreting:			
IgD-CD27^{high}CD38^{high}	0.5 (0.6)	0.5 (0.3)	0.328
Auto-reactive assoc. B-cells:			
CD19^{high}CD21$^{-/low}$	4.7 (2.6)	2.5 (1.0)	0.104
CD19+CD5+	41.1 (19.2)	31.1 (21.9)	0.710

against ANA and c-ANCA were detected, 14 were ANA-positive, 1 c-ANCA positive, and 1 was positive for both c-ANCA and ANA. Interestingly, in contrast to the data we found in the young Tx group, the presence of autoantibodies was not correlated with the percentage of memory T cells (figure 4c) or with the T cell count (figure 4d). In the majority of children, thymic tissue regenerates and thymic output is restored, as measured by CD31 expression in naïve CD4 T cells (figure 4e). In 7 of the 26 patients, regeneration of thymic tissue did not occur as reflected by the low percentage of CD31+ naïve T cells (figure 4e). The difference in thymic output or the absence of functional thymic tissue regeneration did not correlate with autoantibody alterations (figure 4e). Together these data suggest that, although the T cell compartment is restored in these patients over time, induction of altered autoantibody reactivity seems to be more lasting.

Preferential expansion of regulatory T cells in the lymphopenic phase following thymectomy

In the cohort of thymectomized subjects no evidence for clinical autoimmune disease was reported. Peripheral tolerance by regulatory T cells (Treg) could play an important role in preventing altered auto-reactivity from becoming clinically overt autoimmune disease. Concomitant with the decrease in absolute CD4^{+} T-cell numbers after neonatal thymectomy, a significant decrease was seen in Treg numbers, which stabilized in later years (figure 5a). However, the relative proportion of Tregs within the total CD4+ T cell compartment was increased in the first years following thymectomy; this is restored later in life (figure 5b).

This increased proportion of Treg cells could be explained by the preferential proliferation of Tregs compared to other CD4+ T cells that is observed early after thymectomy (figure 5c). Overall, a relative expansion of Tregs is seen in the first years following neonatal thymectomy when T cell lymphopenia is most evident.

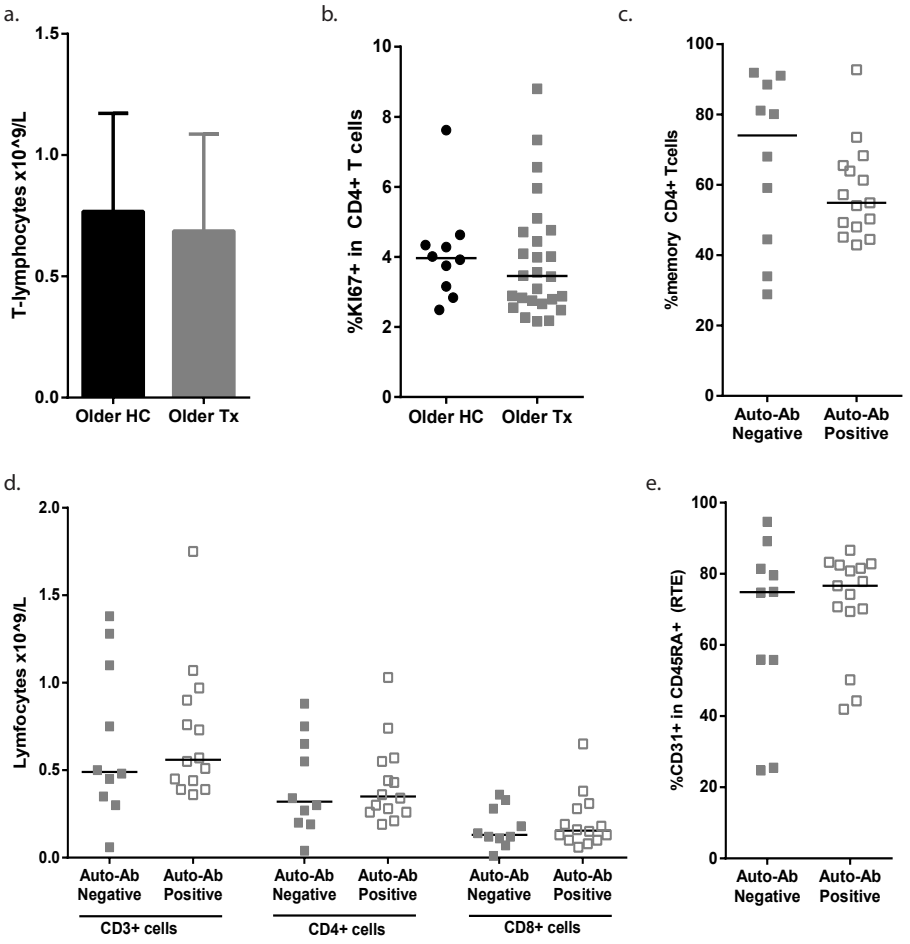

Figure 4. Increased IgG autoantibodies persist later in life independent of T cell number, thymic output and memory CD4 T cells. a) Lymphocyte count in healthy control (Older HC, n=10) and thymectomized (Older Tx, n=24) adolescents (Mean and SD shown) b) Percentage of proliferation (ki-67+) in CD4+ T cells of HC (n=10) and Tx (n=26) c) Percentage memory (CD45RO+) CD4 T cells in auto-antibody positive (ANA and ANCA) and negative Older Tx patients d) Lymphocyte count in auto-antibody positive and negative Tx patients e) Recent thymic emigrants (RTE) in auto-antibody positive and negative Tx patients. Autoantibody negative (n=10), autoantibody positive (n=14-15) median shown.

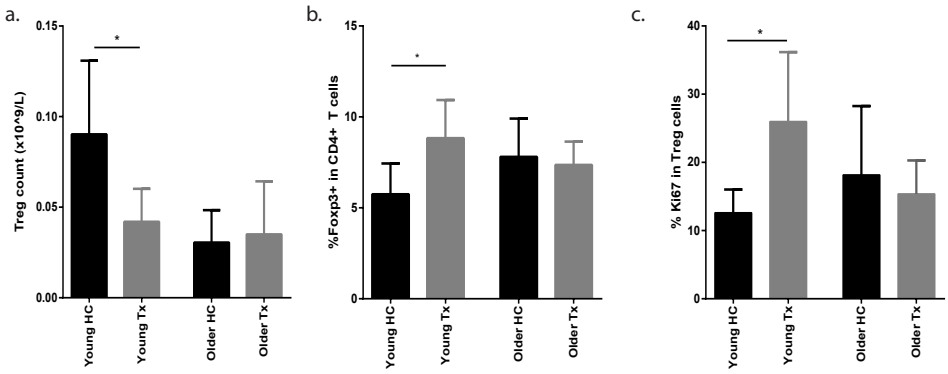

Figure 5. Preferential Treg proliferation during the first years after thymectomy.
a)Treg count in 'young HC' (n=8), 'young Tx'(n=10), 'older HC'(n=10) and 'older Tx'(n=25) b)Percentage Foxp3 positive T-cells in 'young HC'(n=9), 'young Tx'(n=11), 'older HC'(n=10), and 'older Tx'(n=25) c) proliferation of Treg cells in 'young HC'(n=9), 'young Tx'(n=11), 'older HC'(n=10), and 'older Tx'(n=25), Mean and SD shown. * for P<0.05

DISCUSSION

Neonatal thymectomy results in a decrease of T cells and peripheral homeostatic proliferation. [7-9] As peripheral proliferation of T-cells in the lymphopenic setting is partly dependent on recognition of self-peptide/MHC ligands, this is believed to induce an auto-reactive skewed T cell repertoire that could result in increased autoimmunity and autoimmune disease.[34] However, the development of lymphopenia-induced autoimmune disease in humans has been predominantly studied in primary immune deficiencies or autoimmune diseases that most likely have additional impairments in factors regulating auto-reactivity. [35, 36] We show here that neonatal thymectomy results in both quantitative and qualitative changes in autoantibodies; this appears to correlate with increased peripheral T cell expansion in early life.

The antigen microarray assay enables large-scale screening of hundreds of antibody reactivities involved in health and autoimmune diseases.[37] Alterations in both IgM and IgG autoantibody repertoires were detected. IgG autoantibodies have been associated with autoimmune disease; in contrast, IgM autoantibodies have been associated with maintenance of self-tolerance.[16, 17, 30] The presence of IgM polyreactivity has, for example, been correlated to reduced disease severity in lupus patients. [31, 38] Increased IgG reactivity towards cardiolipin and leptintA was detected after neonatal thymectomy. Anti-cardiolipin antibodies are found in several diseases, including antiphospholid syndrome, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis, among others.[39-42] The adipocyte-derived hormone leptin is a pro-inflammatory cytokine that also has a potent role in

mediating many autoimmune diseases [43, 44] In addition, a decreased reactivity of multiple IgM natural autoantibodies was detected in thymectomized children. Some of these IgM antibody reactivities were associated with antigens involved in SLE, such as C1q and single-stranded DNA (DNAss) [38, 45-47] This might indicate that in the early years after neonatal thymectomy, maintenance of self-tolerance is disturbed, which could lead to increased IgG reactivity to self-antigens; our findings suggest that decreased IgM autoantibodies is also a sign of altered self-tolerance. In support of this, about 58% of neonatally thymectomized children manifested auto-reactivity towards ANA (1:100) later in life. This is considerably higher than is reported for healthy adults, where up to 27% can develop autoantibodies to ANA, and at a lower titer (1:40). [48] In any case, the alterations in the autoantibody repertoires that we observed were not accompanied by overt autoimmune disease. This suggests that other mechanisms of self-tolerance or regulation are present to compensate for the altered autoantibody repertoires.

In several autoimmune diseases, the presence of autoantibodies is accompanied with changes in the Tfh and B cell compartment. [32, 33, 49 50-54] Tfh cells are important in providing B cell help to generate specific antibodies.[55 55] In several autoimmune diseases increased Tfh cells are documented and appear to lower the selection threshold and allow survival of low affinity or self-reactive B cell clones.[52] An increased proportion of Tfh cells was present after neonatal thymectomy. However, no drastic changes within the B-cell compartment were apparent after neonatal thymectomy, including loss of CD21 or increased CD5 expression on B-cells reported to be associated with autoimmune disease.[56-58 56-58] The production of free light chains (FLC) is correlated with disease activity in various autoimmune disorders including SLE and RA. [59-61] However the level of both kappa and lambda FLC, nor their ratio differed after neonatal thymectomy. The only difference was a lower total IgG in comparison to healthy controls. This may be influenced by the lower amount of T cells after neonatal thymectomy, necessary for Ig class switching. Together, it seems that the quantity of the B cell response is not affected, as no alterations in the representation of the different B cell subtypes and the global production of immunoglubulins and FLC are seen, but that only the reactivity of the B cell response is affected and skewed towards self-antigens

Even though we detect an increase in auto-reactivity after neonatal thymectomy, we and others have not found an association with clinical autoimmune disease.[62] The development of autoimmune disease is likely the result of failure in several regulatory factors that preserve an adequate homeostasis of self and non-self. Regulatory T cells (Treg) are known to be crucial in the maintenance of peripheral tolerance. After neonatal thymectomy a preferential expansion of Treg was seen in comparison to healthy controls and over non-Treg T cells, which we confirmed in the present cohort.[9] It might be that this preferential proliferation of Treg cells suppresses the development of excessive auto-reactivity in the lymphopenic environment, thereby preventing clinical autoimmune disease.

While neonatal thymectomy results in transiently absent thymopoiesis and thymic tissue function it also entails cardiac surgery. Cardiac surgery has been associated with appearance of autoantibodies, but these responses are usually transient and do not always correlate with the amount of circulating corresponding antigens [63-65] We now show that within the neonatally thymectomized group an association between T cell expansion, and the generation of autoantibodies is apparent. These alterations of auto-reactivity are detected even up to 29 years after neonatal thymectomy. Together this suggests that the long-lasting altered autoantibody response is mainly the consequence of the absence of a thymus and subsequent HP in the years after surgery but may have been fueled by acute trauma during surgery.

Overall, we show that neonatal thymectomy is associated with alterations in the autoantibody repertoire that persist later in life, even when the thymus has regenerated. Surgery necessitating neonatal thymectomy has only been possible for about 30-35 years and autoantibodies can arise long before clinical symptoms develop. [66, 67] For now it is unknown if alterations in auto-reactivity in these thymectomized children will ever or prematurely develop into clinical autoimmune disease later in life but our data indicate that these individuals may be at higher risk. We therefore suggest minimal removal of thymic tissue during cardiac surgery and increased awareness of increased auto-reactivity in this population.

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SUPPLEMENTAL INFORMATION

METHODS (continued)

Flow cytometry antibodies

Antibodies against human CD5 (L17F12), CD8 (Sk-1), CD21 (B-Ly4), CD31 (WM59), CD38 (HIT2), KI-67 (B56), PD-1 (CD279, Clone MIH4), CXCR5 (RF8B2) were from BD Biosciences (San Jose, CA), Goat F(ab')2 IgM and IgG from Southern Biotech(Birmingham, AL), CD19 (J3-119) from Beckman Coulter (Fullerton, CA), CD10 (eBioCB-CALLA), Foxp3 (PCH101) from eBiosciences (San Diego, CA), and CD3 (UCHT1), CD4 (RPA-T4), CD27 (O323), CD45RO (UCHL1), CD45RA (HI100), IL-21 (3A3-N2) from Biolegend (San Diego, CA). Finally, stained mononuclear cells were washed twice in FACS buffer and run on an FACS Canto II and analyzed by using FlowJo software (Treestar).

Auto-antibody measurement

Plasma samples from young thymectomized children (1-5years) were diluted 1:100 and incubated with HEp-20-10 cells and primate liver substrates for ANA analysis, 1:100 with sections of rat kidney for anti-mitochondrial auto-antibodies, 1:100 with rat stomach for smooth muscle auto-antibodies, 1:10 with formaldehyde and formalin fixed neutrophils for detection of anti-neutrophil cytoplasmatic and perinuclear auto-antibodies, 1:100 with primate stomach for detection of anti-gastric parietal cell auto-antibodies, and 1:100 with smooth muscle cells from sections of primate oesophagus. For the older neonatally thymectomized individuals only ANA and ANCA autoantibodies were determined. After washing, attached antibodies were stained using a fluorescein-labeled antibody against human IgG. Two independent raters unaware of subject status evaluated nuclear staining and autoreactivity was rated negative (absent) or positive (weak or stronger staining).

Statistics

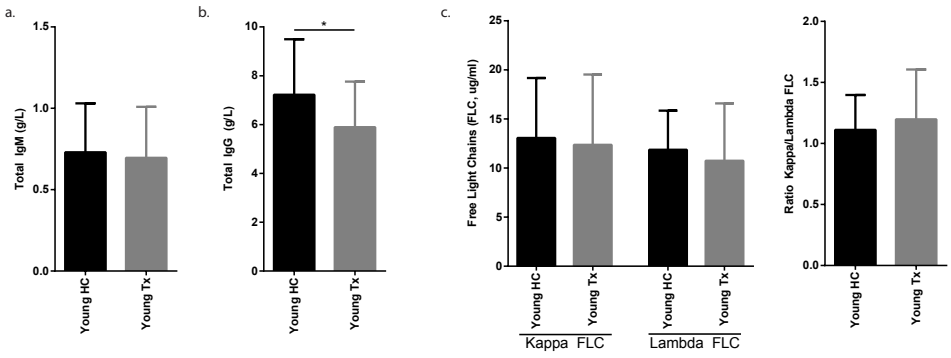
Problematic antigen microarray spots due to smudges or grainy texture were removed manually upon inspection. We then subtracted the background from the foreground for each of the test spot. Antigen reactivity was defined by the mean intensity of 4 replicates binding to that antigen on the microarray; antigen intensities with a mean value lower than zero were marked as missing values. Antigens with less than 70% non-missing values from the total number of samples were removed from further analysis. Each chip was then normalized by its mean reactivity divided by the standard deviation. This was done in order to account for differences in total protein concentrations that affect the background intensity level.

Microarray data was analyzed using R Statistical Software (Core Team R. R: A Language and Environment for Statistical Computing. Vienna; 2013.) and GeneE analysis platform [Gould J (2013). *GENE.E: Interact with GENE-E from R*. R package version 1.8.0, http://www.broadinstitute.org/cancer/software/GENE-E.].

Supplemental table 1. Overview of thymectomized patients (young Tx) and healthy controls (young HC) regarding age and proportion of memory CD4+ T cells.

Patient	Age at thymectomy (d)	Age post-thymectomy (m)	% Memory CD4+ T cells
1	7	15	28,7
2	7	19	34,8
3	11	12	42,6
		23	50,6
4	21	23	37,9
5	10	24	41,6
6	9	25	28,5
7	7	27	48,3
8	5	24	72,2
9	30	32	53,2
		69	65,9
10	7	25	23,5
11	n/a	36	10,2
12	n/a	25	25,2
13	n/a	31	13,1
14	n/a	47	22,7
15	n/a	28	9,9
16	n/a	52	20,3
17	n/a	14	9,5
18	n/a	15	9,9
19	n/a	33	24,2

Patients 1-10 are young Tx, patients 11-19 are young HC. D, days; m,months



Supplemental figure 1. Immunoglobulin production is relatively unaltered after neonatal thymectomy a)Total IgM (g/L) in plasma of HC (n=31) and Tx (n=25) b)Total IgG (g/L) in plasma of HC(n=31) and Tx(n=25) c)left panel, amount of kappa and lambda free light chain in HC (n=12) and Tx (n=20). right panel ratio of Kappa/lambda FLC for HC and Tx. * for P<0.05

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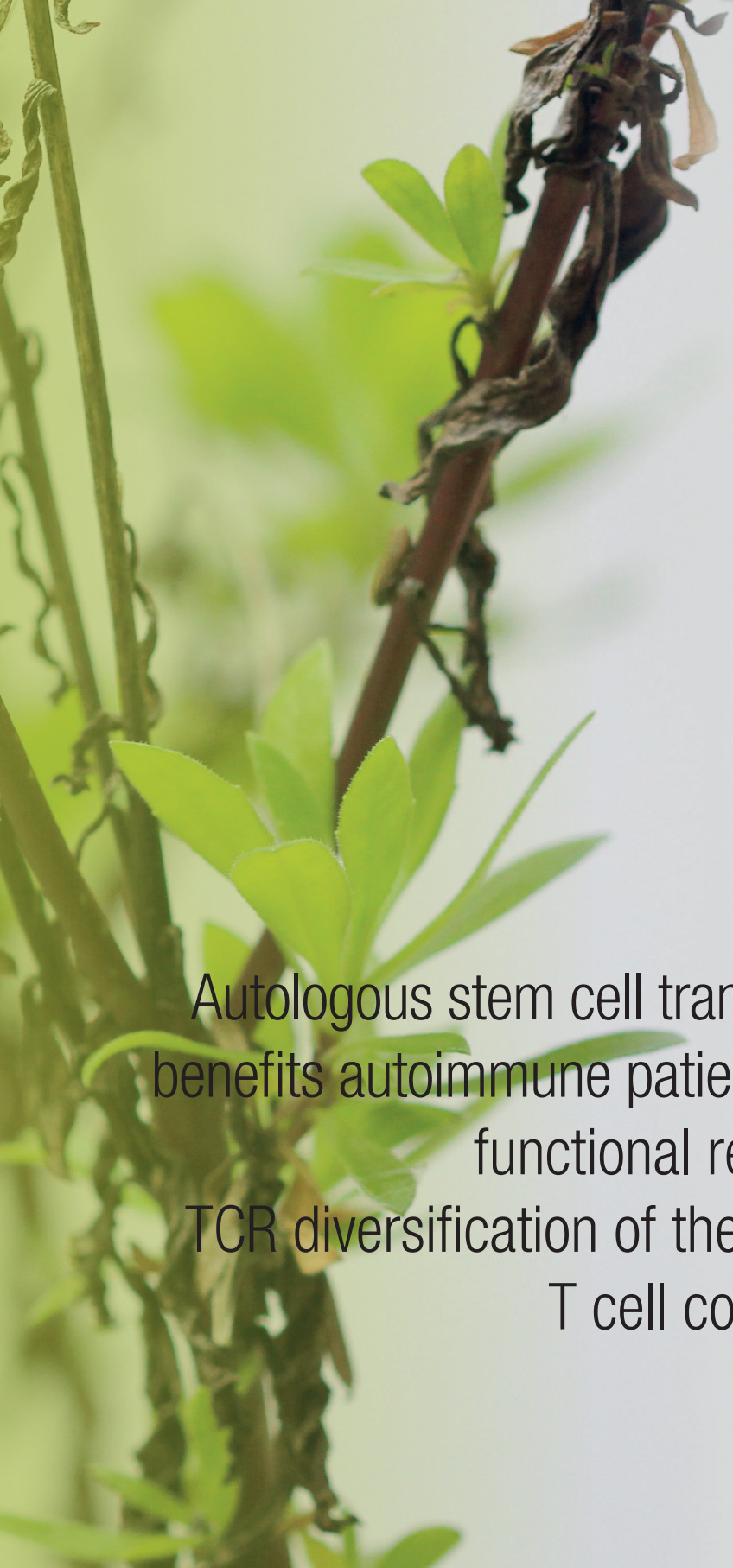

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Autologous stem cell transplantation benefits autoimmune patients through functional renewal and TCR diversification of the regulatory T cell compartment

Submitted

ABSTRACT

Autologous stem cell transplantation (aSCT) is a last resort treatment for patients with refractory autoimmune diseases. The underlying working mechanisms remain largely unknown. Regulatory T cells (Treg) are thought to be important for disease remission after aSCT. Eliciting the role of donor and host Treg in aSCT is however not possible in humans due to autologous nature of the intervention. Therefore, we investigated their role during immune reconstitution and re-establishment of immune tolerance and their therapeutic potential following autologous bone marrow transplantation (aBMT) in a proteoglycan-induced arthritis (PGIA) mouse model. In addition, we determined Treg T cell receptor CDR3 diversity before and after aSCT in patients with refractory autoimmune disease. In the PGIA aBMT model, after an initial predominance of host Treg, graft-derived Treg started dominating and displayed a more stable phenotype with better suppressive capacity. In patients, a striking lack of diversity of the Treg repertoire was found before aSCT, which was ameliorated after aSCT confirming reset of the Treg compartment following aSCT. In the mouse model a therapeutic approach was initiated by infusing extra Foxp3^{GFP+}Treg during aBMT. Infusion of Foxp3^{GFP+}Treg did not elicit additional clinical improvement but conversely delayed reconstitution of the graft-derived T cell compartment.

These data indicate that aSCT-mediated amelioration of autoimmune disease involves renewal of the Treg pool. In addition, infusion of extra Treg during aBMT results in a delayed reconstitution of T-cell compartments. Treg therapy may therefore hamper development of long-term tolerance and should therefore be approached with caution in the clinical autologous setting.

INTRODUCTION

Even in the era of biologicals, some patients with autoimmune diseases remain therapy refractory. For these severely ill patients, autologous stem cell transplantation (aSCT) has shown to be the only treatment able to induce long-term drug- and symptom-free remission.[1-5] As stem cell therapy is not expected to be a mainstream treatment, studies to explore how stem cell therapy resets the immune balance are pivotal. Insights in this mechanism may yield new therapeutic options that achieve the same goal (disease and medication free remission, but with reduced toxicity).

The general concept of aSCT for AID is that immune reconstitution after profound lymphodepletion and immune suppression leads to restoration of the immune balance and regain of immune tolerance. [6] How the procedure is exactly rewiring a faulty immune system is still unknown. For example, it is not clear which cells must be destroyed prior to transplantation, nor is it known which ones keep disease under control afterward.[7] Immediately after re-infusion through aSCT, the lymphopenic environment induces selective expansion and activation of the few (potentially auto-reactive) T cell clones that have survived the conditioning regimen or may have been retransferred with the graft [8]. Therefore lymphopenia-induced proliferation and activation of T cells may pose a risk of loss of self-tolerance early after aSCT. The second phase of T cell reconstitution starts when T cells develop in the thymus and naive T cells are introduced in the periphery,[9] contributing to the resetting of the immune system.[8] In humans, $CD4^+$ T cell receptor (TCR) sequencing has confirmed selective expansion of several TCR clones directly after treatment, followed by broadening of the total TCR repertoire during follow up due to thymus output.[10;11] During both reconstitution phases regulatory T cells (Treg) may be essential to control T cell reconstitution and activation but little is known about the renewal of the Treg compartment.

Treg are thought to be important players for disease remission in aSCT-treated AID, especially in the lymphopenic reconstitution phase when the delicate immune balance has to be re-established [12;13]. In children with refractory JIA it has been shown that prior to aSCT Treg blood levels are decreased, while after aSCT, Treg levels are comparable to healthy controls [14-16]. Furthermore, we have shown in an experimental arthritis model that depletion of Treg post autologous bone marrow transplantation (aBMT) results in an improvement of arthritis scores, suggesting a pivotal early role for Treg for disease remission[17]. Expansion of the Treg compartment following aSCT starts with lymphopenia induced proliferation followed by thymic output of stem cell derived Treg.[13] What remains to be established is the relative contribution of stem cell graft-derived Treg versus conditioning-survived host Treg in controlling T cell activation and disease remission. This knowledge is of increasing importance regarding the recent introduction of less toxic non-myeloablative conditioning regimens that may limit the role of the graft.[7] We hypothesized that graft-

derived Treg are the main contributors in the restoration of a functional Treg compartment following aSCT. In the human transplantation setting it is impossible to study the relative contribution due to the autologous nature of the treatment. Here, we tested our hypothesis, distinguishing "host" and "donor" Treg dynamics and function following aBMT in an experimental AID setting by using a congenic marker. To translate our findings to the human setting we performed Treg T cell receptor β chain variability (TCRBV) CDR3 sequencing in autoimmune patients undergoing aSCT. In addition, we addressed whether infusion of additional Treg during aBMT led to suppression of residual potentially harmful effector T cells.

MATERIAL AND METHODS

Mice

CBy.PL(B6)-Thy1a.ScrJ (CD90.1, Jackson Laboratory) mice were used for bone marrow grafts or served as recipients when indicated. Foxp3-IRES-GFP mice (CD90.2) were obtained for the isolation of regulatory T cells (Treg). Female retired breeder Balb/c mice (CD90.2) were acquired from Charles River Laboratory and offspring of crossed CD90.1 and CD90.2 mice were bred in house. Both served as recipients. All mice are on a Balb/c background.

Mice were kept in the Utrecht University Animal Facility under regular conditions. After autologous bone marrow transplantation (aBMT), recipient mice were accommodated under sterile conditions. All experiments were approved by the Animal Experiment Ethical Committee of the University of Utrecht.

Induction and assessment of arthritis

Both have been described in detail earlier.[18;19] In short, two and 5 weeks before aBMT, arthritis was induced by two intraperitoneal injections of proteoglycan (PG) together with adjuvant dimethyldioctadecylammonium bromide (DDA). The onset and severity of arthritis were assessed three times a week in a blinded fashion by a visual scoring system.[17]

Treatment protocols

Autologous bone marrow transplantation

Two weeks after the second PG/DDA injection, mice were lethally irradiated (7.5 Gy). Within 6 hours of irradiation, mice were injected with $2x10^6$ bone marrow (BM) cells. As the genetic background of syngeneic and autologous BM was identical, the term autologous BMT (aBMT) is used throughout this paper.

Bone marrow suspension
BM was acquired by flushing the tibia and femur bones. BM cells were resuspended in 200 µl 0.2% bovine serum albumin before injection into the tail vein. The mean percentage of T cells present in BM was 2,05 ± 0,3% SD.

Infusion of extra Treg
After sacrificing Foxp3-IRES-GFP mice, spleens and joint-draining (inguinal and popliteal) lymph nodes (LN) were harvested. Cells were harvested by pushing them through a cell strainer after which $CD4^+$ T cells were positively selected via magnetic cell sorting beads (L3T4, Miltenyi Biotec). Treg were stained and isolated as $TCR\beta^+CD4^+CD25^+GFP^+$ T cells on a FACS Aria II (BD Biosciences).

In vitro assays
Flow cytometry
One, three, five and seven weeks after aBMT thymus, spleen, blood and joint-draining LN cells were harvested when indicated. In addition, seven weeks post transplantation, synovial fluid was obtained by needle aspiration of the knee and ankle joints. Cells were stained with antibodies against TCR-β (clone H57-597), CD25 (clone PC61), CD90.1 (clone OX-7), Ki-67 (clone B56), CD45RB (clone 16A) and CD44 (clone IM7) (BD Biosciences, San Jose, CA, USA), CD4 (clone RM4-5), CD90.2 (clone 53-2.1), Foxp3 (clone FJK-16a) (eBioscience, San Diego, CA, USA)
To calculate absolute T cell numbers, BD Tru Count Beads were added just before the acquisition of the samples. Data were analyzed with FACS Diva (6.13, BD Biosciences).

Foxp3 demethylation assay
Seven weeks after aBMT, spleen-derived Treg were isolated with flow cytometry as $TCR\beta^+CD4^+CD25^+$ cells and distinguished for host cells ($CD90.2^+$) or donor cells ($CD90.1^+$). DNA from these cells was isolated using the DNeasy Blood & Tissue kit (Qiagen) and bisulfate converted applying the EpiTect Bisulfate kit (Qiagen Hilden) following the manufactures recommendations. Demethylation assay was performed by EPIONTIS GmbH (Berlin, Germany).[20]

Suppression assay
Seven weeks after aBMT, spleen cells were harvested and enriched for $CD4^+$ T cells by CD4 microbeads isolation. The negative fraction was used as antigen-presenting cells in the culture. Host and donor Treg were separated with flow cytometry. CFSE-labelled $TCR\beta^+CD4^+CD25^-$ spleen cells from healthy Balb/c mice were used as effector T cells. Treg were added in different ratios to the effector T cells. Soluble anti-CD3 (1µg/ml, clone 145-2c11, BD Pharmingen, San Diego) was used as stimulus. At day 4, the proliferation of

effector T cells was analyzed with flow cytometry. In Figure 5B, host and donor Treg originated from CD90.1 origin and Foxp3-IRES-GFP Treg expressed CD90.2. For the Foxp3 demethylation, suppression and qPCR assays only a relatively small amount of material was available. Therefore, mice of the same groups were combined to obtain enough material for the assays.

Real-time quantitative polymerase chain reaction (qPCR)
After RNA extraction from lysed host and donor Treg real-time qPCR was performed for for Helios, Neuropillin-1, interleukin-10 and mGAPDH (primers see Table S1). Gene expression was calculated as CT ($(2^{-dCT)}$*100) with mGAPDH as housekeeping gene.

Next generation T cell receptor (TCR) β chain variability (TCRBV) spectratyping
Frozen PBMC's from aSCT treated patients with juvenile dermatomyositis (patients 1 and 2) and juvenile idiopathic arthritis (patients 3 and 4) were thawed. Treg ($CD3CD4CD25^{high}CD127^{low}$) and non-Treg ($CD3CD4CD25^{low/medium}CD127^{medium/high}$) were sorted (obtained Treg between 9.700 and 48.500 cells and non-Treg between 190.000 and $1x10^6$ cells) and frozen at -80°C. On average, 74,1 % of sorted Treg expressed FOXP3.
Total RNA was isolated using the RNeasy Mini Kit (Qiagen) for cell fractions >$0.2x10^6$ cells, the RNeasy Micro Kit (Qiagen) for fractions <$0.2x10^6$ cells following the instructions of the manufacturer. cDNA was synthesized using the SMARTer™ RACE cDNA Amplification Kit (Clontech). Amplification of the TCRBV region was performed using previously described primers and amplification protocols [21]. PCR products were analysed with a QIAxcel Advanced System (Qiagen). Upon successful amplification, end repair was performed with the ClaSeek Library Preparation Kit, Illumina compatible (Thermo Scientific). Subsequently, TruSeq Barcode adapters (Illumina) were ligated using the ClaSeek Ligation Mix (Thermo Scientific) according the recommendations of the manufacturer. Cleanup of the samples was performed with The Agencourt AMPure XP system (Beckman Coulter). Next generation sequencing was performed on an Illumina MiSeq system 500 (2x250bp) (Illumina).
Sequencing data were analyzed with the MiTCR program [22]. The MiTCR output file was used to calculated the Simpson's Index (D), $D = \frac{\sum n(n-1)}{N(N-1)}$, in which n= total number of specific sequence and N= the total number of all sequences. Data are presented by the Simpson's Index of Diversity (Di), . Di = 0 indicates no diversity, whereas Di = 1 represents maximal diversity.

Multiplex assay
Spleen cells were obtained seven weeks after transplantation. Supernatants of a 96-hour culture of $2x10^5$ spleen cells with the addition of 1μg/ml anti-CD3 were analyzed with a

mouse cytokine multiplex kit (Biorad, CA, USA). Analysis was performed using the Bioplex manager software v4.1 (Biorad).

Statistical analysis

To identify differences between aBMT-treated PGIA animals and untreated PGIA animals, the Mann-Whitney U test was used. To achieve normal distribution for cytokine data, logarithmic transformation was performed before applying Mann-Whitney U test. Significant differences between host and donor cells were tested by using Wilcoxon matched-pairs signed rank test. All data are presented as the mean + SEM values (error bars). P values less than 0.05 were considered significant. Statistical analysis was performed using IBM SPSS Statistics Version 20.

RESULTS

aBMT renews the Treg compartment with donor-derived Treg that directly home to the site of inflammation

To investigate the reconstitution of T cells after aBMT, a congenic marker was used to distinguish host T cells (cells that survived conditioning, $CD90.2^+$) from donor T cells (cells originating from the graft, $CD90.1^+$). One week after aBMT, most $TCR\beta^+CD4^+CD25^+Foxp3^+$ Treg present in thymus and spleen were of host origin (Figure 1A, left and middle graphs). During the reconstitution phase three weeks following transplantation, the thymus harboured more donor-derived Treg (50,3% ± 9,1 SEM) compared to the spleen (37,5% ± 4,8 SEM) and LN (38,6 ± 4,1 SEM, Figure 1A). Seven weeks post aBMT, the majority of Treg were of donor origin in all investigated organs (Figure 1A). As expected, similar reconstitution dynamics in absolute number of donor-derived Treg was seen post-aBMT, while host cells remained relatively stable over time (Figure S1A).

Comparable results were obtained for total $CD4^+$ T cells (Figure S1B and S1C). In thymus and spleen, host $CD4^+$ T cells were most abundant directly after aBMT. During reconstitution, the majority of donor $CD4^+$ T cells was first detectable in the thymus, followed by spleen and LN in percentages and absolute numbers.

To investigate if donor Treg could home to the site of inflammation, synovial fluid was obtained of knee and ankle joints. The majority of synovial fluid Treg was donor-derived (Figure 1B).

These results indicate that aBMT induces graft-derived renewal of the $CD4^+$ T cell and Treg compartments through thymus-derived donor T cell reconstitution. Furthermore, donor-derived Treg home to the joint, the site of inflammation.

Donor-derived Treg have a more naive and functional phenotype than host-derived Treg.

Next, we explored the Treg phenotype following aBMT. Three weeks after aBMT, the minority of both host- and donor-derived Treg expressed a naive phenotype ($CD45RB^{+}CD44^{-}$, Figure 2A). Seven weeks post transplantation, the percentage of naive Treg increased in both host and donor compartments, with a slightly higher rise in the donor-derived population (Figure 2A). Both Treg populations showed increased proliferation compared to Treg of PGIA control animals (Figure 2B) and this increase was still observed for donor Treg 7 weeks after aBMT. Furthermore, at seven weeks Ki-67 expression was significantly higher in donor Treg than host Treg.

Helios and Neuropilin-1 have been described as markers for thymic-derived Treg.[23-25] The amount of Helios nor Neuropilin-1 mRNA expression, associated with thymic-derived Treg (Figure 2C left and right panels), did not differ between donor and host Treg. In terms of functionality, significantly increased IL-10 mRNA expression was seen in donor-derived Treg (Figure 2D).

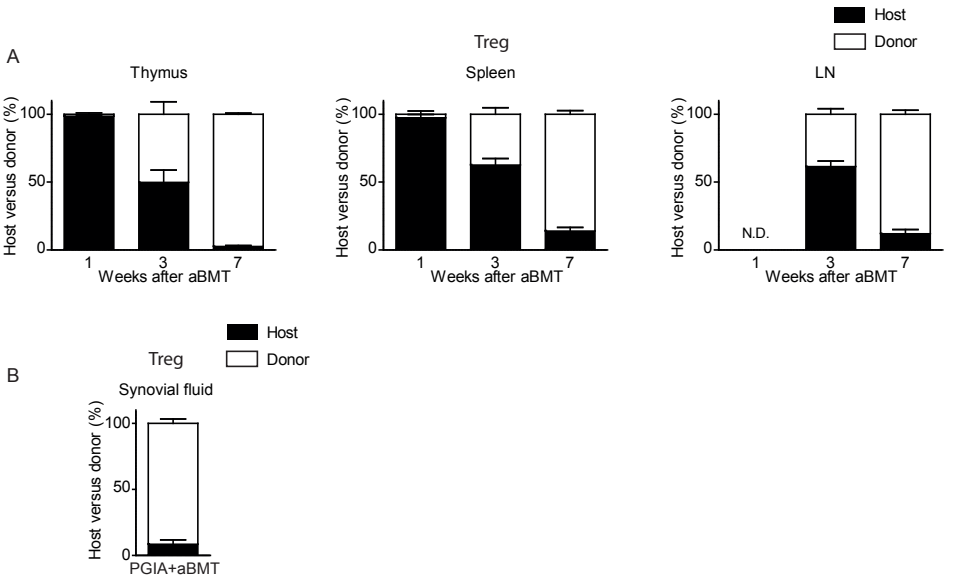

Figure 1. aBMT for arthritis renews the regulatory T cell compartment with donor-derived Treg.
Host T cells (black bar, CD90.2+) were distinguished from donor T cells (white bar, CD90.1+)) using a different congenic marker. After transplantation, thymus, spleen and lymph nodes (LN) were analyzed at week 1, 3 and 7 for the presence of host and donor Treg (TCRβ+CD4+CD25+Foxp3+). Results shown are from two combined experiments. (A) Percentage of host and donor Treg. Thymus 1 week N=3, 3 weeks N=6, 7 weeks N=8, spleen 1 week N=2, 3 weeks N=6, 7 weeks N=8, LN 3 weeks N=6, 7 weeks N=8. (B) Host and donor Treg distribution in synovial fluid, 7 weeks post aBMT, N=2. All results shown are in percentages (± SEM values). N.D.; not determined due to lack of cells.

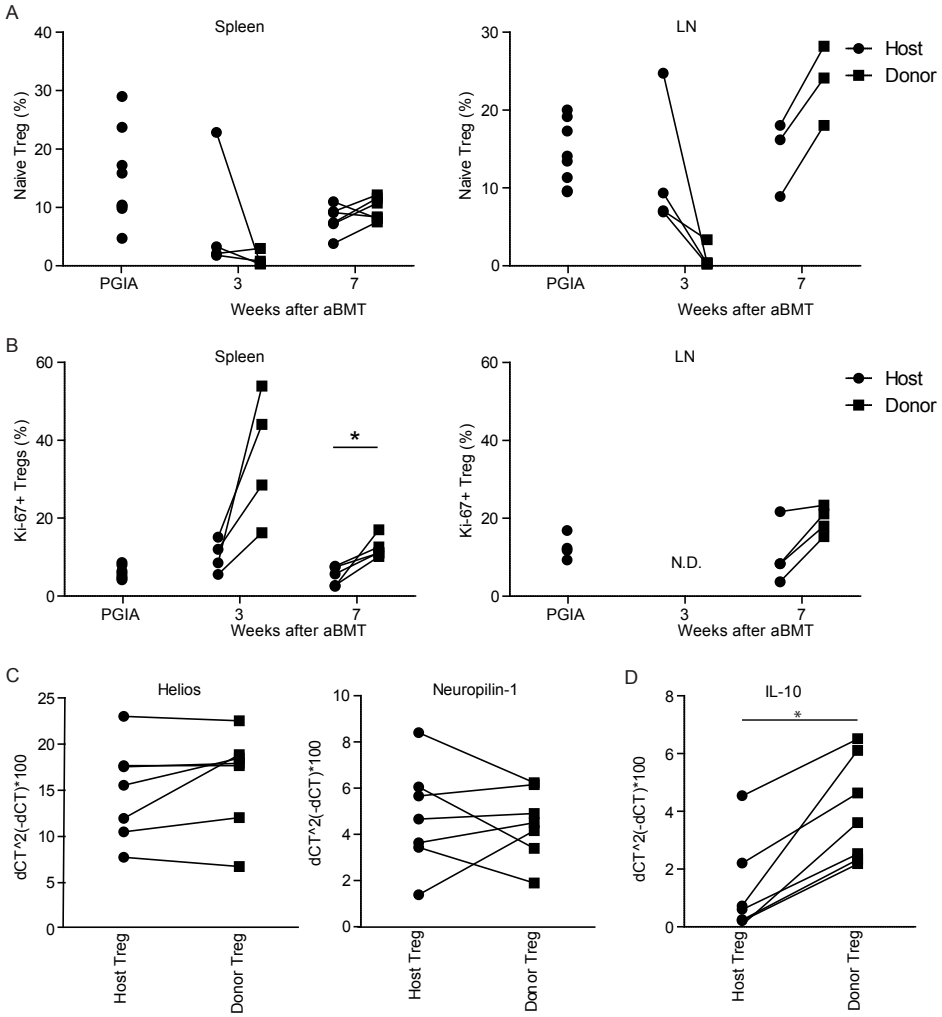

Figure 2. Post aBMT, donor Treg show a naive, proliferative and anti- inflammatory phenotype. Host (circles) and donor (squares) Treg (TCRβ+CD4+CD25+Foxp3+) were isolated from spleen and LN 3 and 7 weeks post aBMT from aBMT-treated mice with arthritis and PGIA controls (pooled data from 3 and 7 weeks). Results shown are from two combined experiments. (A) Percentages host versus donor naive Treg (CD45RBhigh, CD44low). Spleen (left panel) PGIA control N=7, aBMT-treated mice N=4 at 3 weeks and N=5 at 7 weeks post aBMT. LN (right panel) PGIA control N=8, aBMT-treated mice N=4 at 3 weeks, N=3 at 7 weeks. (B) Percentages host versus donor proliferative Treg (Ki-67+). Spleen (left panel) PGIA control N=3, aBMT-treated mice N=4 at 3 weeks, N=5 at 7 weeks. LN (right panel) PGIA control N=3, aBMT-treated animals N=4 at 7 weeks. (C) Host versus donor-derived spleen Treg from PGIA- aBMT treated animals. 7 weeks after aBMT, spleen Treg (TCRβ+CD4+CD25+) were sorted and relative mRNA expression of Helios (C, left panel, N=7, 10 mice) and Neuropillin-1 (C, right panel, N=7, 10 mice) was measured by qPCR. (D) Relative expression of anti-inflammatory cytokine marker IL-10 mRNA in spleen Treg (N=7, 10 mice). * indicates p-value <0.05 calculated using Wilcoxon rank test. N.D.; not determined due to lack of cells.

These data indicate that initially both donor Treg and host Treg expand vigorously after aBMT. At a later stage, naive, donor-derived Treg develop with a possible suppressive phenotype.

aBMT induces a stable and functional donor-derived Treg compartment

The demethylation status from the Treg-specific demethylated region (TSDR),[26] a highly conserved element within the first intron of the *Foxp3* gene was investigated to asses stability of the Treg (Figure 3A). The level of Foxp3 TSDR demethylation of donor Treg was comparable to Treg of PGIA control mice. However, host-origin Treg showed less TSDR demethylation compared to donor-origin Treg, suggesting that donor Treg are more stable. Subsequently, the suppressive function of host and donor Treg was determined in a suppression assay. Donor-derived Treg were efficient in suppressing proliferation of effector

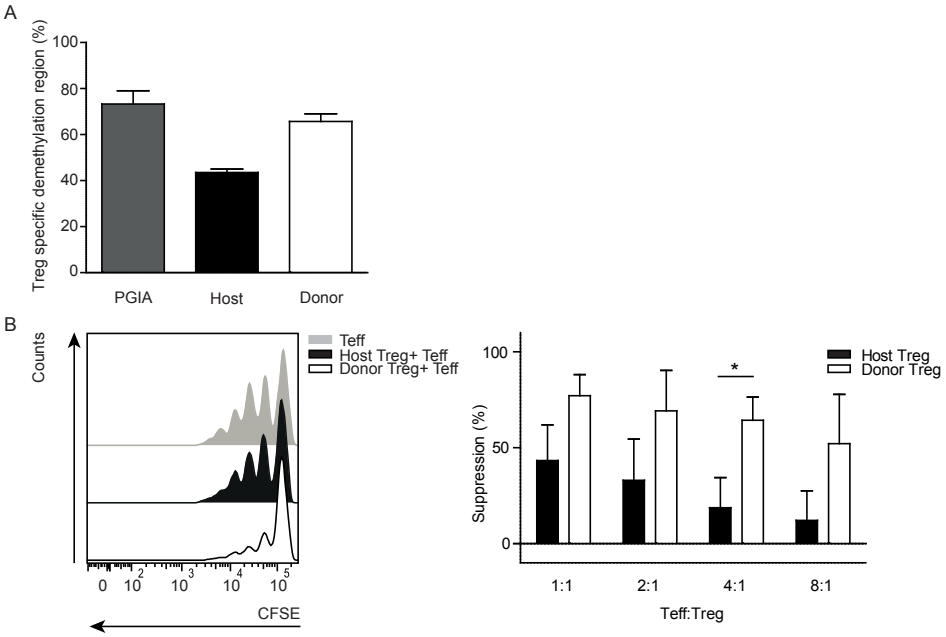

Figure 3. Donor-derived Treg are stable and functional. (A) Demethylation status of the Foxp3 gene (Treg specific demethylation region, TSDR) was analysed from spleen host and donor Treg (TCRβ+CD4+CD25+), isolated 7 weeks post aBMT from PGIA control and aBMT-treated animals. PGIA control N=4 mice pooled in 2 groups before measurement aBMT-treated mice N=5 (3 PGIA- induced of which 2 were pooled in 1 group, and 2 non-arthritic induced animals pooled into 1 group). (B) Left panel. Representative CFSE dilution histogram of suppressive capacity of FACSsorted host and donor Treg compared to proliferative capacity of effector T cells of healthy mice. Right panel. Summarized suppressive capacity of host and donor Treg after aBMT at different Teff:Treg ratios 1:1, 2:1, 4:1 and 8:1. aBMT-treated animals N=6 (4 PGIA-induced and 2 non-induced animals). Treg were paired before suppression assay. * indicates p-value <0.05, host compared versus donor calculated using Mann-Whitney U test.

T cells (Figure 3B). Although host-derived Treg were also able to suppress effector T cell proliferation, a significant difference was observed in the suppressive function of donor versus host Treg, showing that donor Treg were superior suppressors (Figure 3B).
These results reveal that a stable and functional donor-derived Treg compartment is formed post aBMT.

Oligoclonal Treg receptor repertoire becomes more diverse after successful autologous stem cell transplantation (aSCT) in refractory autoimmune patients
To translate our findings on the renewal of the Treg compartment to the human setting we performed next generation TCRBV CDR3 sequencing of Treg and $CD4^+$ non-Treg T cells in refractory autoimmune patients undergoing aSCT. Following aSCT, patients 1, 2 and 3 had accomplished disease remission without medication (longest follow up 3,5 years), whereas patient 4 experienced disease relapse 6 months after transplantation.
In healthy controls the Treg TCR repertoire has been found to be as diverse as the non-Treg compartment.[27-29] Here, strikingly, the number of unique Treg TCR sequences (n) and TCR diversity (Di) prior to aSCT found for patients 1-4 was extremely low ranging from n= 4 to 19 and Di= 0,114 to 0,687 (Figure 4A and B). In contrast, the non-Treg showed a higher number of unique TCR sequences and higher Di calculations (Figure 4A and B). After aSCT, the number and diversity of Treg TCR sequences increased over time (patient 1 and 2, Figure 4A and B). The non-Treg compartments showed an increase in TCR sequences as well, although the Di increase was not as pronounced as in the Treg (Figure 4B). In the patient with disease relapse (patient 4) the Treg TCR repertoire even increased in oligoclonality following transplantation (n=9, Di=0,071) (Figure 4A and B). None of the predominant Treg and non-Treg TCR sequences existing prior to transplantation were retraceable in the same population after treatment (cut off 20 reads per sequence). All together, we conclude that successful aSCT leads to a renewed and more diverse Treg TCR repertoire.

During bone marrow transplantation injected $Foxp3^{GFP+}$ Treg are retraceable and remain functional over time in mice
Based on our findings in patients indicating the importance of renewal/diversification of the Treg compartment for disease remission after aSCT, we questioned if aSCT results could be improved by Treg infusion together with the graft for the suppression of pro-inflammatory residual effector T cells. To explore clinical improvement with the immediate presence of Treg after aBMT, $Foxp3^{GFP+}$ Treg were added in different numbers (250.000 or 500.000 cells) to the graft.
Longitudinal blood punctures were performed to examine if the infused $Foxp3^{GFP+}$ Treg were retraceable during the observation period. Three weeks post aBMT, $Foxp3^{GFP+}$ Treg comprised the majority of the total $CD4^+$ T cell compartment. However, this prominence

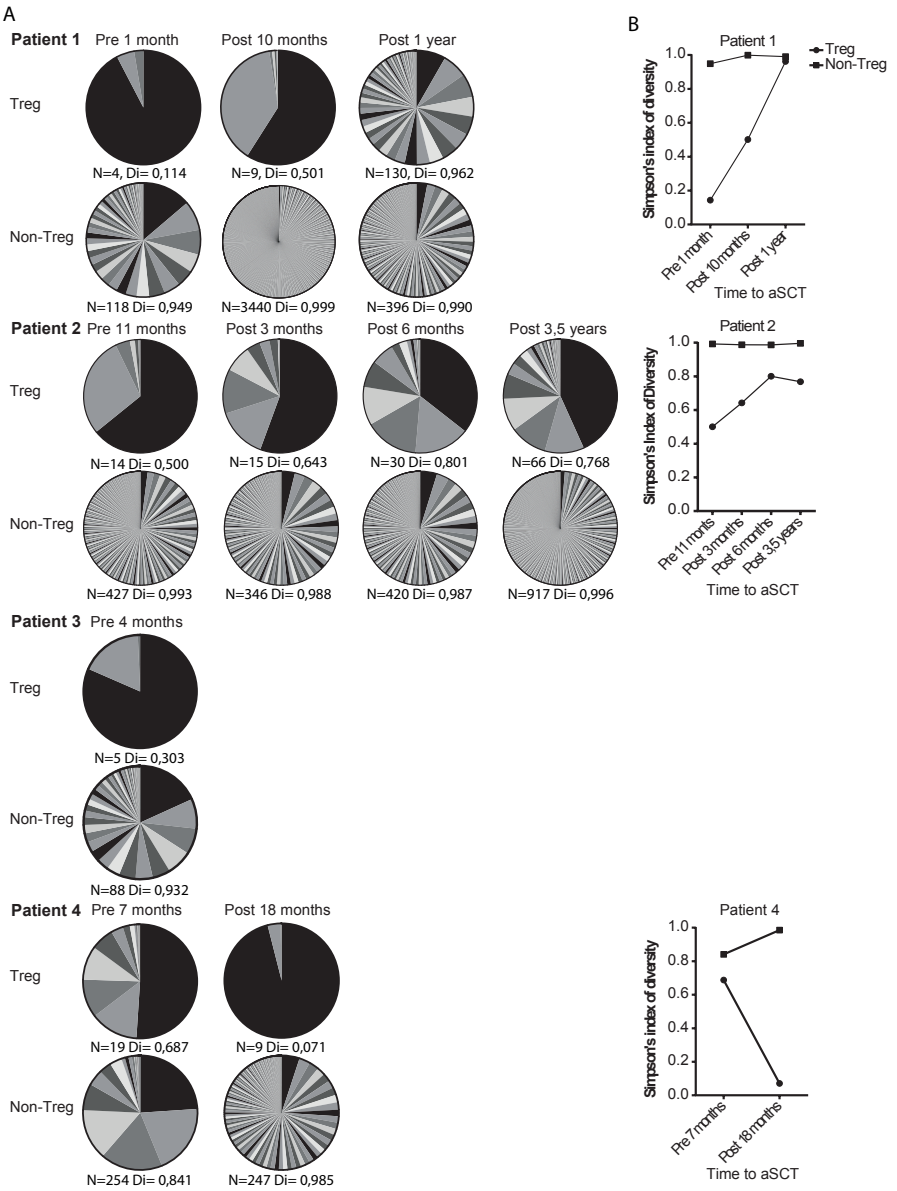

Figure 4. Successful aSCT leads to a renewed and more diverse Treg TCR repertoire. (A) Treg and non-Treg from different blood collections of 4 aSCT-treated patients with refractory autoimmune disease were sorted. These cell samples were used for T cell receptor TCRBV CDR3 sequencing. Per patient each time point of blood sampling is shown. Per time point, two pie charts show the number and abundance of TCR sequences found for the Treg and non-Treg sorted from that cell sample. Colour overlap between different pie charts does not represent the same TCR sequence. N represents the number of different TCR sequences found per sample, Di indicates the sample's diversity (0= no diversity, 1= maximal diversity). (B) For patient 1, 2 and 4 the changes in Di prior to aSCT and during follow up is shown in graphs.

disappeared during the following weeks and did not differ between the two Foxp3^{GFP+} Treg treatment groups (Figure 5A, left panel). The decrease in the percentage of Foxp3^{GFP+} Treg coincided with an increase of the donor-derived CD4^{+} T cell compartment (Figure 5A middle and right panel).

The Foxp3^{GFP+} Treg present at the end of the observation period remained functional as shown in the suppression assay (Figure 5B). The suppressive capacity of the Foxp3^{GFP+} Treg was similar compared to total non-GFP^{+} Treg of the same mice and of regular transplanted animals. These results demonstrate that additional injected Foxp3^{GFP+} Treg survive, expand and remain functional.

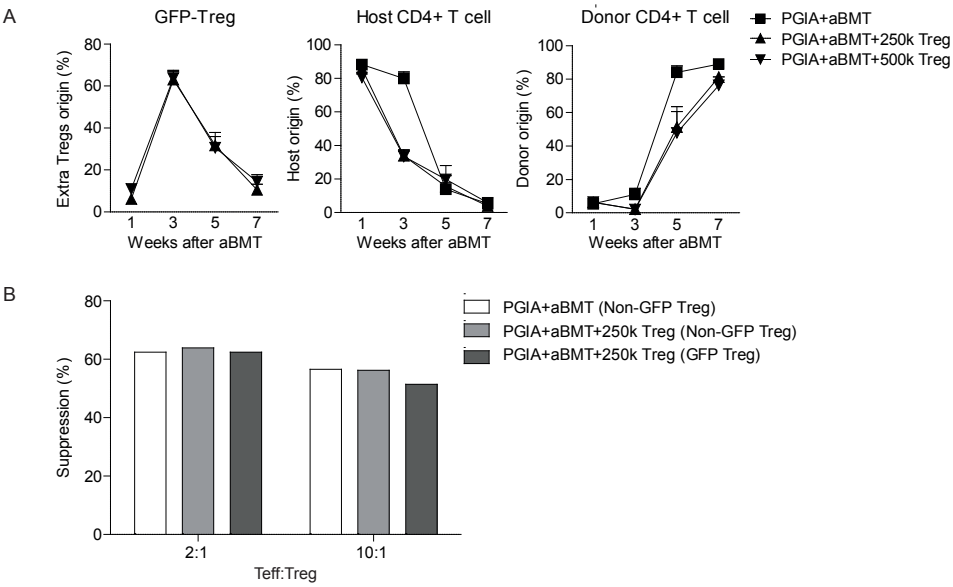

Figure 5. Extra infused Foxp3GFP+ Treg at time of aBMT are retraceable during follow-up and remain functional. Arthritic mice were treated with 2x10^6 bone marrow cells (aBMT). In addition, two treatment groups received 250.000 or 500.000, GFP+ Treg in the bone marrow graft. Blood was analyzed 1, 3, 5 and 7 weeks after aBMT. (A, Left panel) Percentages of additional infused Foxp3GFP+ Treg, (A, middle panel) percentages of host TCRβ +CD4+ T cells and (A, right panel) percentages of donor TCRβ+CD4+ T cells. PGIA+aBMT N=4, PGIA+aBMT+250k Treg N=5, PGIA+aBMT+500k Treg N=5. (B) Suppression assay of Treg from PGIA+aBMT treated mice (white bar), non-GFP Treg from PGIA+aBMT+ 250k Treg treated mice (light gray), and Foxp3GFP+ Treg from aBMT+250k Treg treated mice (dark gray). Treg were added in 1:2 and 1:10 ratios to healthy effector T cells. Treg were pooled per treatment group before adding to the effector T cells. PGIA-aBMT N=2 mice, PGIA+aBMT+250k Treg N=3.

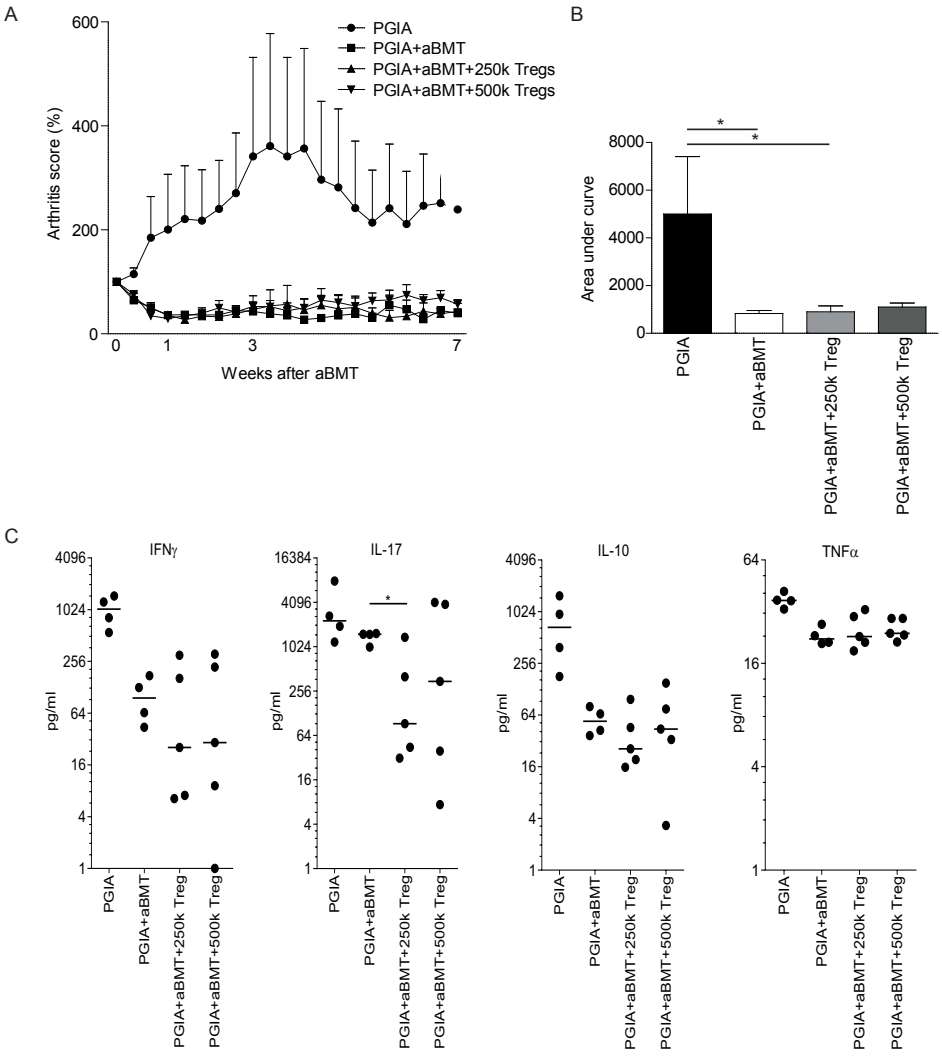

Figure 6. Additional Treg in the graft reduces T cell produced pro-inflammatory cytokines but does not lead to a better clinical outcome. (A) Arthritis scores after transplantation. Arthritis scores were set to 100% on the day of transplantation and the subsequent clinical effect was expressed as a percentage of the score at the time of transplantation. Mean arthritis scores are shown (+ SEM error bars). Data are representative for two individually performed experiments. (B) Area under the arthritis score curve during the 7 week follow-up. Mean AUC + SEM error bars are shown. PGIA (black bar, N=4), PGIA+aBMT, (white bar, N=4), PGIA+aBMT+250k Treg (gray bar, N=5), PGIA+aBMT+500k Treg (dark gray bar, N=5). * indicates p-value <0.05 comparing to PGIA control group calculated using Mann-Whitney U test. (C) Spleen cells were isolated 7 weeks after aBMT and cultured in culture medium with the addition of antiCD3 (1ug/ml) for 96 hours. Supernatants were collected and analyzed with Multiplex Immuno Assay for IFNγ, IL- 17, IL-10 and TNFα production. PGIA N=4, PGIA+aBMT N=4, PGIA+aBMT+250k Treg N=5 and PGIA+aBMT+500k Treg N=5. * indicates p-value <0.05 compared to PGIA+aBMT using Mann Whitney U test.

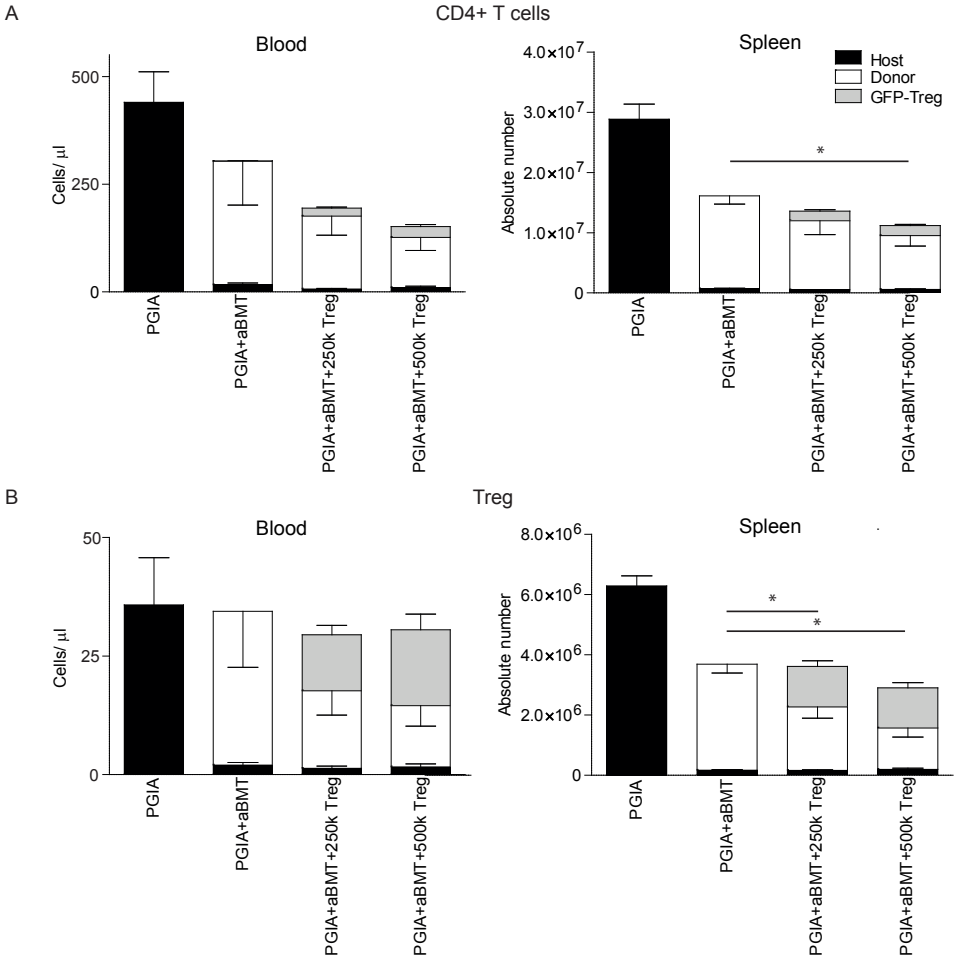

Figure 7. Addition of extra Foxp3GFP+ Treg results in a reconstitution delay of donor BM-derived Treg. A and B. 7 weeks post aBMT, blood and spleen cells were isolated and stained with congenic markers to distinguish extra injected Foxp3GFP+ Treg (grey bar), host (black bar) and donor (white bar) for CD4+ T cells (TCRβ+CD4+) and Treg (TCRβ+CD4+CD25+Foxp3+). CD4+ T cell reconstitution in blood (A, left panel), CD4+ T cell reconstitution in spleen (A, right panel). Treg reconstitution in blood (B, left panel), Treg reconstitution in spleen (B, right panel). All figures show absolute numbers, ± SEM error bars. PGIA N=3, PGIA+aBMT N=4, PGIA+aBMT+250k Treg N=5 and PGIA+aBMT+500k Treg N=5. * $p<0.05$ for donor cell compartment.

The addition of Foxp3^{GFP+} Treg to the graft does not improve clinical outcome, but decreases pro-inflammatory cytokine production

Next, we questioned whether the addition of these Treg led to an improvement of the clinical outcome. The arthritis scores of mice treated with Foxp3^{GFP+} Treg did not differ compared to mice treated with a regular aBMT (Figure 6A). To evaluate the clinical effect during the entire observation period, the area under the curve was calculated (Figure 6B). In both PGIA+aBMT and PGIA+aBMT+250k Foxp3^{GFP+} Treg treated groups, significant improvement in suppressing arthritis severity was seen compared to the untreated PGIA group. In the PGIA+aBMT+500k Foxp3^{GFP+}Treg dosing group a similar improvement was seen, although not significant.

Cytokine production of each treatment group was assessed by ex vivo stimulation of splenocytes. All aBMT treated groups showed lower pro-inflammatory cytokine production compared to the PGIA group, which is in line with the clinical amelioration. In addition, both Foxp3^{GFP+} Treg treated groups demonstrated a tendency of decreased IFNγ and significantly decreased IL-17 compared to regular transplanted animals (Figure 6C). For IL-10 and TNFα no difference was observed between the three aBMT treatment groups.

These data demonstrate that the addition of Foxp3^{GFP+} Treg to the graft did not lead to a better clinical outcome, despite reduced pro-inflammatory cytokine production in these groups.

Infusion of extra Foxp3^{GFP+} Treg with the aBMT graft causes a delay in donor-derived T cell and Treg reconstitution

Treg are known to suppress T cell proliferation. Therefore, we hypothesized that the additional Treg present in the graft could also influence T cell reconstitution following aBMT. Seven weeks post aBMT, the total number of CD4$^+$ T cells in blood and spleen of the PGIA+aBMT+ Foxp3^{GFP+} Treg treated groups was smaller compared to PGIA+aBMT treated animals (Figure 7A). Although the total number of Treg remained similar in the three different treatment groups (Figure 7B), donor-derived Treg numbers were smaller in the Treg-treated groups, with a significant reduction in the 500k treated group (Figure 7B). The delay in donor Treg reconstitution was already evident at 5 weeks post aBMT (Figure S2). Together these data indicate that the addition of Foxp3^{GFP+} Treg to the graft leads to a delay in donor-derived CD4$^+$ T cell and Treg reconstitution.

DISCUSSION

Autologous stem cell transplantation is the only treatment that can induce long-term drug free disease remission in refractory AID. Treg are thought to be important players for remission after aSCT. In the clinical setting, exploring immunological mechanisms that

underlie disease remission is difficult as no distinction can be made between conditioning survivor cells and graft-derived cells. With the use of a congenic T cell marker and the PGIA model, we investigated "host" and "donor" Treg dynamics following aBMT. Here, the results indicate that aBMT, in addition to renewal of the effector T cell compartment,[8] also renews the Treg compartment with donor-derived Treg that were superior in function to the remaining host Treg.

Most donor-derived Treg were initially found in the thymus, followed by the peripheral organs. In the first weeks after aBMT, host Treg proliferation occurred with a decrease in naive Treg followed by an increase in naive donor-derived Treg. These observations suggest that in the current model donor-derived Treg originate from precursor cells that develop in the thymus and not by peripheral proliferation of donor-derived $CD4^+$ T cells in the graft. To further explore this in humans, TCRBV CDR3 sequencing was performed in aSCT-treated autoimmune patient samples. We found a remarkable Treg oligoclonality in patients before aSCT, which was not present in the non-Treg $CD4^+$ T cell compartment. Limited data are available on clonal diversity of the TCR repertoire of Treg but in healthy individuals the $CD4^+FOXP3^+$ Treg repertoire seems as diverse as the $CD4^+$ non-Treg repertoire.[27-29] Our data are the first to show that chronic inflammation and Treg oligoclonality exist simultaneously, and to a much larger extent than the $CD4^+$ non-Treg compartment. Whether this contributes to autoimmune inflammation remains to be investigated. In the aSCT setting we found no T cell receptor sequence overlap between pre-existing Treg and non-Treg versus post-treatment Treg and non-Treg samples. In addition there was a clear broadening of the Treg TCR repertoire following successful aSCT and induction of disease remission. Together this suggests that post aSCT there is a renewal and diversification of the naturally occurring thymic-derived Treg compartment. Muraro and colleagues have recently demonstrated renewal of the general CD4 TCR repertoire in MS patients undergoing aSCT. This was in contrast to $CD8^+$ T cells, which showed expansion of existing TCRs.[10] The TCR diversity of the non-Treg compartment also expanded after aSCT, although not as striking as the Treg compartment, indicating that the Treg compartment is more affected. In conclusion, thymic reconstitution of both $CD4^+$ T cell and Treg compartments seem to play a pivotal role following aSCT. In JIA patients, 90% of the disease relapses is found in the first year post aSCT and this indeed correlates with the absence of thymus involvement in T cell reconstitution.[15;30]

It is known that lymphopenic conditions are able to reduce Foxp3 expression of Treg and thereby their suppressive function.[31-34] In addition, host-derived Treg survived a strong conditioning regimen when present post aBMT. These events may together lead to activated Treg that (temporarily) lose their suppressive function after transplantation. This could explain the functional difference between host and donor Treg.

Given the importance of the Treg compartment in preventing disease relapse following aBMT [17], we initiated a therapeutic approach by infusing Treg at the time of aBMT to control

early Teff activation and to potentially improve clinical outcome. We show that despite a decreased pro-inflammatory T cell cytokine production, no additional clinical improvement was detected. Importantly, infusion of extra Treg resulted in a delayed donor-derived T cell and Treg reconstitution. This delayed donor naive $CD4^+$ T cell/Treg reconstitution may abolish the potential beneficial effect of the additional Foxp3 Treg. Even though the total number of Treg were equal in all aBMT treated groups after transplantation, it is likely that the extra-infused Treg will disappear after a certain period [35]. Remaining Treg will then be essential for retaining disease remission and their numbers maybe insufficient due to delayed reconstitution induced by the infused Treg.[36] Peripheral Treg have indeed recently been shown to inhibit their precursors in the thymus resulting in less Treg output by the thymus,[37] suggesting that extra infused Treg may delay donor Treg reconstitution via inhibition of the thymic output. Together our data suggest that there is a delicate balance between the addition of Treg to the graft and optimizing reconstitution of the donor-derived T cell compartment. Murine and human studies have investigated Treg infusions to prevent graft versus host disease (GvHD) in the allogenic SCT setting. These studies observed no T cell reconstitution delay and even showed enhanced T cell reconstitution post treatment.[38;39] This may underline a difference between autologous and allogeneic stem cell transplantation.

In summary, we here showed that aBMT induces a functional and stable donor-derived Treg compartment in an experimental arthritis model. In addition, total Treg TCR repertoire renewal was observed after successful aSCT in humans. With the renewed interest in aSCT due to the use of non-myeloablative conditioning [7], it is important to realize that replacing the immune system may be crucial for inducing and maintaining long-term immune tolerance. More knowledge about the immunological mechanisms that lead to renewed immune balance will help us adjust current treatment regimens and develop new approaches with similar outcome as aSCT but with less severe side effects.

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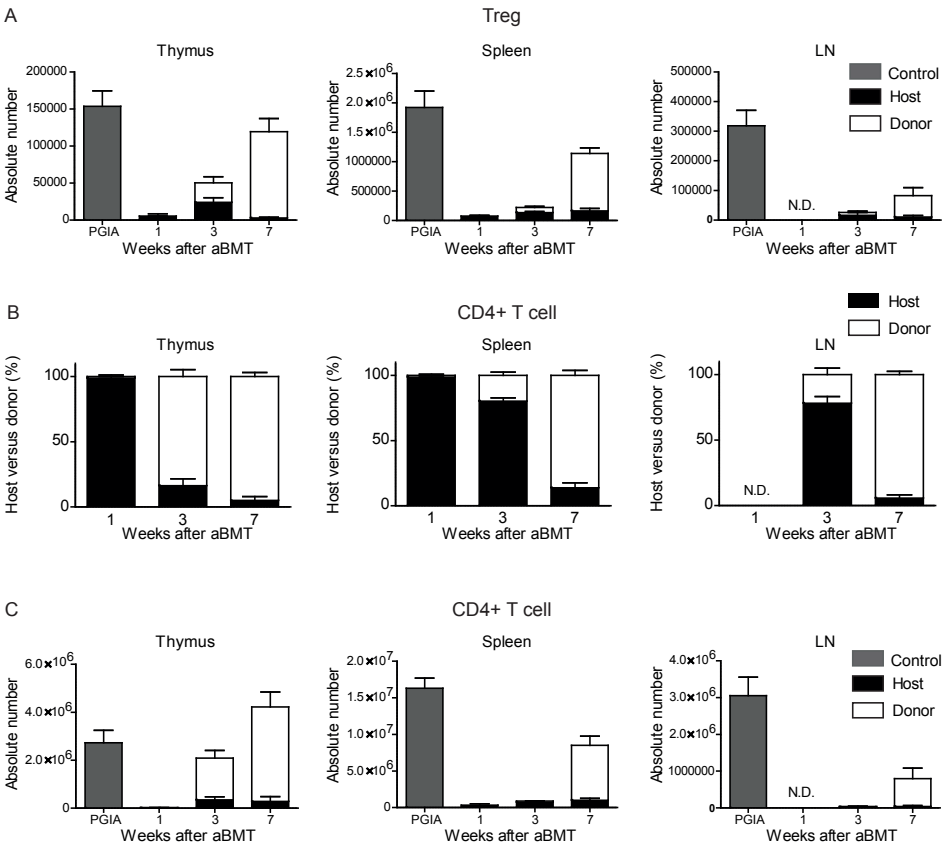
SUPPLEMENTAL INFORMATION

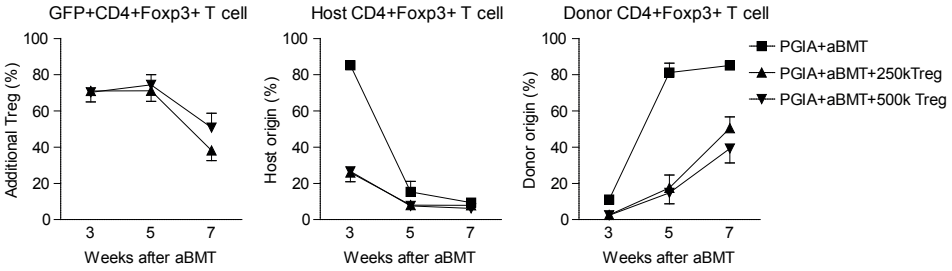
Supplemental table 1. Primer sequences

Target	Primer sequence
Demethylation-specific	
Forward primer	X:7161172-95:1
Reverse primer	X:7161337-66:1
Hydrolysis probe	X:7161251-80:1
Methylation-specific	
Forward primer	X:7161176-95:1
Reverse primer	X:7161336-60:1
Hydrolysis probe	X:7161257-78:1
Helios	
Forward primer	5'- TCA CAA CTA TCT CCA GAA TGT CAG C -3'
Reverse primer	5'- AGG CGG TAC ATG GTG ACT CAT -3'
Neuropilin-1	
Forward primer	5'- GAC AAA TGT GGC GGG ACC ATA -3'
Reverse primer	5'- TGG ATT AGC CAT TCA CAC TTC TC-3'
Interleukin-10	
Forward primer	5'- ACT CTT CAC CTG CTC CAC TG -3'
Reverse primer	5'- GCT ATG CTG CCT GCT CTT AC -3'
mGAPDH	
Forward primer	5'- GCC TTC CGT GTT CCT ACC C -3'
Reverse primer	5'- TGC CTG CTT CAC CAC CTT C -3'



Supplementary Figure 1. Absolute number of donor CD4⁺ T cells and Treg increase rapidly post aBMT. A, B and C. Arthritis was induced by two injections of PG in DDA, 5 and 2 weeks before aBMT. At week 0, Balb/c mice (CD90.2) received a lethal irradiation dose of 7.5 Gy followed by intravenous injection of $2x10^6$ bone marrow cells from donor (CD90.1) mice. 1, 3 and 7 weeks after transplantation, thymus, spleen and LN were analyzed for the presence of host (CD90.2, black bar) and donor (CD90.1, white bar) CD4⁺ T cells (TCR⁺CD4⁺) and Treg (TCRβ⁺CD4⁺CD25⁺Foxp3⁺). Results shown are from two combined experiments. Data obtained from PGIA control mice on different timepoints are pooled. (**A**) Absolute number of host and donor Treg. PGIA N=8, Thymus 1 week N=3, 3 weeks N=6, 7 weeks N=8, spleen 1 week N=2, 3 weeks N=6, 7 weeks N=8, LN 3 weeks N=6, 7 weeks N=8. (**B**) Percentage of host and donor CD4⁺ T cells. Thymus 1 week N=3, 3 weeks N=7, 7 weeks N=8, spleen 1 week N=4, 3 weeks N=7, 7 weeks N=9, LN 3 weeks N=6, 7 weeks N=6. (**C**) Absolute number of host and donor CD4⁺ T cells. PGIA N=8, Thymus 1 week N=3, 3 weeks N=7, 7 weeks N=8, spleen 1 week N=3, 3 weeks N=7, 7 weeks N=9, LN 3 weeks N=6, 7 weeks N=6. + SEM bars are shown.

A



Supplementary Figure 2. Donor Treg reconstitution is delayed in blood after the addition of extra GFP^{+} Treg in the graft

Arthritis was induced by two injections of PG in DDA 5 and 2 weeks before aBMT. At week 0, CD90.1/CD90.2 Balb/c mice received a lethal irradiation dose of 7.5 Gy followed by 2x10^{6} CD90.1 bone marrow cells (aBMT) infusion. In addition two treatment groups received 250.000 or 500.000 CD90.2, Foxp3^{GFP+} Treg. Blood was drawn 3, 5 and 7 weeks after aBMT. Treg (TCRβ^{+}CD4^{+}Foxp3^{+}) were stained with congenic markers to distinguish host, donor and extra infused Treg. (**l**eft panel) Percentages of additional infused Foxp3^{GFP+} Treg, (middle panel) percentages of host Treg and (right panel) donor Treg of the total Treg population. PGIA+aBMT (squares) N=4, PGIA+aBMT+250k Treg (pointing-up triangles) N=5, PGIA+aBMT+500k Treg (pointing-down triangles) N=5.

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7

Autologous Stem Cell Transplantation Restores Immune Tolerance in Experimental Arthritis by Renewal and Modulation of the Teff Cell Compartment

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Objective
Autologous stem cell transplantation (ASCT) induces long-term drug-free disease remission in patients with juvenile idiopathic arthritis. This study was undertaken to further unravel the immunologic mechanisms underlying ASCT by using a mouse model of proteoglycan-induced arthritis (PGIA).

Methods
For initiation of PGIA, BALB/c mice received 2 intraperitoneal injections of human PG in a synthetic adjuvant on days 0 and 21. Five weeks after the first immunization, the mice were exposed to total body irradiation (7.5 Gy) and received (un)manipulated bone marrow (BM) grafts from mice with PGIA. Clinical scores, T cell reconstitution, (antigen-specific) T cell cytokine production, and intracellular cytokine expression were determined following autologous BM transplantation (ABMT).

Results
ABMT resulted in amelioration and stabilization of arthritis scores. BM grafts containing T cells and T cell–depleted grafts provided the same clinical benefit, with similar reductions in PG-induced T cell proliferation and the number of PG-specific autoantibodies. In vivo reexposure to PG did not exacerbate disease. Following ABMT, basal levels of disease-associated proinflammatory cytokines (interferon-γ [IFNγ], interleukin-17 [IL-17], and tumor necrosis factor α [TNFα]) were reduced. In addition, restimulation of T cells with PG induced a strong reduction in disease-associated proinflammatory cytokine production. Finally, although the remaining host T cells displayed a proinflammatory phenotype following ABMT, IFNγ, IL-17, and TNFα production by the newly reconstituted donor-derived T cells was significantly lower.

Conclusion
Taken together, our data suggest that ABMT restores immune tolerance by renewal and modulation of the Teff cell compartment, leading to a strong reduction in proinflammatory (self antigen–specific) T cell cytokine production.

Juvenile idiopathic arthritis (JIA) and rheumatoid arthritis are autoimmune diseases that often lead to major disability. The introduction of biologic agents such as anti-tumor necrosis factor α (anti-TNFα) has been a major step forward in controlling disease symptoms. In general, however, these treatments cannot induce drug-free disease remission. Furthermore, some patients fail to respond to conventional treatment or become unresponsive to treatment over time. For severely ill patients with JIA, autologous stem cell transplantation (ASCT) has proven to be an effective last resort [1,2]. ASCT induces drug-free disease remission in a majority of patients during followup of up to 80 months posttransplantation [3].

The drug-free disease remission achieved by the majority of patients undergoing ASCT suggests that ASCT can, at least temporarily, restore immune tolerance in JIA. However, the underlying mechanisms are largely unknown. Data from our previous study of patients with JIA suggested that both renewal of the Treg cell compartment and reprogramming of Teff cells may play a role [4]. Unfortunately, T cell reconstitution after ASCT in humans cannot be monitored, because residual T cells and autologous graft–derived T cells are not distinguishable. A better understanding of the mechanisms would greatly favor the development of new treatment strategies that aim at not only immune suppression but also restoring immune tolerance.

Although it remains to be elucidated what underlies the impressive success of ASCT, immune reconstitution after profound depletion appears to favor development of tolerance over pathogenic immunity. Immediately after reinfusion with ASCT, the lymphopenic environment induces selective expansion and activation of the few T cell clones present. These T cells either survived the conditioning regimen [5] or may have been retransferred with the graft and are potentially autoreactive. Therefore, lymphopenia-induced proliferation and activation of T cells may pose a risk of loss of self-tolerance early after ASCT. During this period, the presence of Treg cells may be essential to control T cell reconstitution and activation. At a later stage, the CD4+ T cell pool is further reconstituted by naive recent thymic emigrants, which is crucial for diversification of the T cell repertoire following ASCT [6] and may also play a role in the reestablishment of immune tolerance. Thus, the antigen specificity, differentiation, and function of the reconstituting T cells appear to be decisive for the efficacy of ASCT and warrant further investigation.

To elucidate this process, we developed an experimental model for ASCT in a mouse model of proteoglycan-induced arthritis (PGIA). PGIA is studied extensively, is clinically, immunologically, and histopathologically similar to human arthritis, and has a chronic relapsing–remitting course [7,8]. Using this model, we previously demonstrated a crucial role for Treg cells in the recovery phase after autologous bone marrow transplantation (ABMT) [9]. Here, we explore the influence of ABMT on the Teff cell compartment and demonstrate that ABMT-induced renewal of the T cell compartment leads to a strong reduction in proinflammatory (self antigen–specific) T cell responses.

MATERIALS AND METHODS

Mice

Female retired breeder BALB/c mice were obtained from Charles River Deutschland. CBy.PL(B6)Thy1a.ScrJ mice, ages 7–10 weeks, were obtained from The Jackson Laboratory and served as donors for ABMT when indicated. The mice were maintained under standard conditions. After ABMT, recipient mice were housed under sterile conditions. All experiments were approved by the Animal Experiment Committee of the Faculty of Veterinary Medicine at Utrecht University.

Induction and assessment of arthritis

Arthritis was induced in BALB/c mice by 2 intraperitoneal injections of PG (400 ug) in 2 mg dimethyldioctadecylammonium bromide (DDA; Sigma Aldrich) 2 weeks and 5 weeks before bone marrow transplantation. PG was purified as described previously[7,8]. The onset and severity of arthritis were assessed 3 times each week using a visual scoring system, as described previously[9]. Briefly, the degree of joint swelling, redness, and deformation of each paw was scored on a scale of 0–4 to determine a total arthritis score (maximum possible score of 16 per mouse).

Treatment protocols

Two weeks after the second injection of PG in DDA, the recipient mice in which arthritis developed received a lethal dose (7.5 Gy) of total body irradiation. Next, ABMT was performed by intravenous injection of 2 x 10^6 BM cells obtained from syngeneic donor mice. Bone marrow was harvested by flushing the tibia and femur with Iscove's modified Dulbecco's medium including penicillin and streptomycin. BM cells (2 x 10^6) were resuspended in 200 ul 0.2% bovine serum albumin before intravenous injection into the tail vein. The mean percentage of T cells in BM grafts was 3.2%. For specific experiments, T cells were depleted from the BM graft using anti-mouse CD4 and anti-mouse CD8 MACS MicroBeads (Miltenyi Biotec). The T cell–depleted BM cells contained 0.67% (CD3+) T cells (data not shown).

Approximately 4–5 weeks after ABMT was performed, untreated arthritic mice and those that received unmanipulated grafts were given a booster dose of PG (400 ug administered intraperitoneally). The clinical response in these mice was compared with that in untreated arthritic mice and those that received unmanipulated grafts but were given an intraperitoneal injection of phosphate buffered saline (PBS) instead of PG.

In vitro assays

T cell proliferation

Seven weeks following ABMT, spleens and axial lymph nodes were harvested. Cells (2 X 10^5/well) were cultured in Iscove's modified Dulbecco's medium for 120 hours in the absence or presence of 10 ug/ml PG. During the last 16–18 hours of culture, 1 uCi 3H-thymidine (Amersham) was added to each well. Proliferative responses were calculated as the median 3H-thymidine incorporation (counts per minute) of triplicate wells.

Cytokine production

One, three, and seven weeks after ABMT, spleen cells (2 x 10^5) were cultured in culture medium for 96 hours in the presence of 10 ug/ml PG or 1 ug/ml soluble anti-CD3 (clone 145-2C11; BD PharMingen). Cytokine profiles were measured using a Mouse Cytokine Multiplex Kit (Bio-Rad) according to the manufacturer's instruction.

Flow cytometry and IgG1 enzyme-linked immunosorbent assay

For flow cytometric analysis, spleen, lymph node, and synovial fluid cells were stained for T cell receptor β (TCRβ), CD90.1, Ki-67 (BD PharMingen), CD4, CD90.2, FoxP3 (eBioscience), CD45RB, and CD44 (BioLegend). For IgG1 enzyme-linked immunosorbent assay, plates were coated with PG (0.5 ug/well) and blocked with 1.5% milk in PBS. Sera were added at a 1:100.000 dilution, and PG-specific antibodies were determined using peroxidase-conjugated rat anti-mouse IgG1 monoclonal antibody (clone X56; 1:1000). Serum antibody levels were calculated relative to mouse serum immunoglobulin fractions in the pooled sera of control mice.

Intracellular cytokine production

Seven weeks after ABMT, spleen and lymph node cells (5 X 10^5) were cultured with phorbol myristate acetate (25 ng/ml) and ionomycin (500 ng/ml; Calbiochem) for 5–6 hours. After 1 hour, GolgiStop (BD Biosciences) was added to the cultures. Cells were stained for anti-mouse TCRβ, CD4, CD90.1, TNFα, interleukin-17 (IL-17) (all from BD PharMingen), and interferon-γ (IFN γ) antibodies (eBioscience).

Statistical analysis

The Mann-Whitney U test was used to determine differences between untreated and ABMT- treated mice with PGIA. To achieve normal distribution for cytokine data, logarithmic transformation was performed before applying the Mann-Whitney U test. Wilcoxon's matched pairs signed rank test was performed to determine significant differences between host cells and donor cells. All data are presented as the mean ± SEM. P values less than 0.05 were considered significant.

RESULTS

Decreased disease activity using T cell–depleted or unmanipulated BM grafts for ABMT

Because the results of clinical studies have suggested that the presence of low numbers of potentially self-reactive memory T cells in an infused stem cell graft may pose a risk of disease relapse, we performed ABMT using T cell depleted BM grafts and unmanipulated BM grafts. Both treatments resulted in a decrease in arthritis scores, which remained low compared with the scores of untreated mice with PGIA until the end of the observation period (Figure 1A). The area under the arthritis score curve was also significantly lower in both BMT treatment groups (T cell depleted and unmanipulated grafts) compared with untreated mice with PGIA (Figure 1A).

To determine whether T cell depletion of the graft led to a difference in PG-specific responses, spleen and lymph node cells were restimulated with PG. After in vitro exposure to PG, proliferation was reduced in both treatment groups compared with the untreated control group (Figure 1B). In addition, the reductions in PG-specific IgG1 antibody levels following ABMT were similar in mice that received unmanipulated grafts and those that received CD4+ T cell–depleted grafts (Figure 1C), suggesting that the presence of low numbers of T cells in the graft does not influence treatment outcome. Next, to determine persistence of tolerance to the disease antigen, a booster dose of PG was administered intraperitoneally to ABMT-treated and control mice with PGIA, 10 weeks after the first injection of PG in DDA. In control mice with PGIA, in vivo reexposure to PG resulted in increased arthritis scores, whereas arthritis scores in ABMT-treated mice remained stable (Figure 1D), indicating that ABMT induces in vivo tolerance.

Effect of ABMT on basal levels of antigen-specific T cell production of IFNγ, IL-17, and TNFα

Following ABMT, the number of CD4+ T cells was clearly reduced, and cells with a memory phenotype predominated (Figure 2A). The antigen specificity, differentiation, and function of reconstituting T cells are likely decisive for the efficacy of ABMT in autoimmune diseases. In untreated control mice, basal levels of IFNγ, IL-17, and TNFα were observed after culturing spleen cells without any stimulus (Figure 2B). After stimulation with PG, the production of IFNγ, IL-17, and TNFα almost doubled in the cell cultures of untreated mice (Figure 2C). In contrast, splenocytes from mice that underwent ABMT did not show IFNγ, IL-17, and TNFα production when cultured in medium alone. In addition, no production of IFNγ, IL-17, or TNFα could be measured following culture with PG. This absence of PG-induced production of proinflammatory cytokines was observed 1, 3, and 7 weeks following ABMT, suggesting long-term suppression of PG-specific T cell responses. The lack of cytokine production by splenocytes derived from mice treated with ABMT was not

the result of a general impairment in cytokine production by these T cells, because nonspecific anti-CD3 stimulation induced similar levels of IFNγ, IL-17, and TNFα in the control and ABMT groups (Figure 2D). Taken together, these results show that ABMT leads to a less inflammatory environment and a strong reduction in self antigen–induced cytokine production.

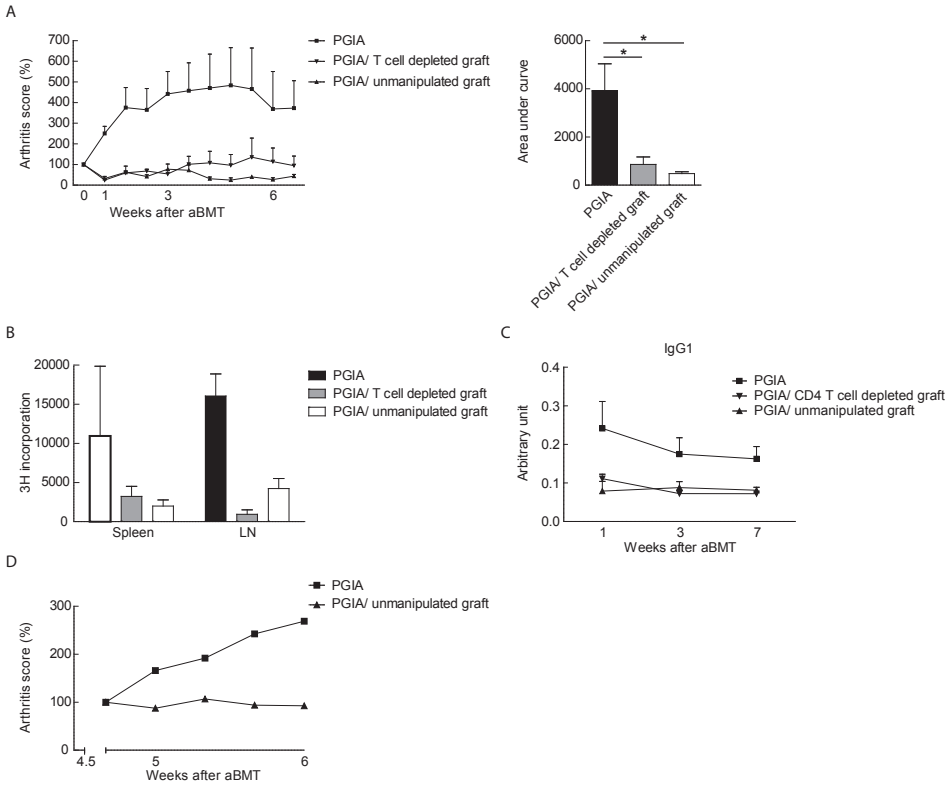
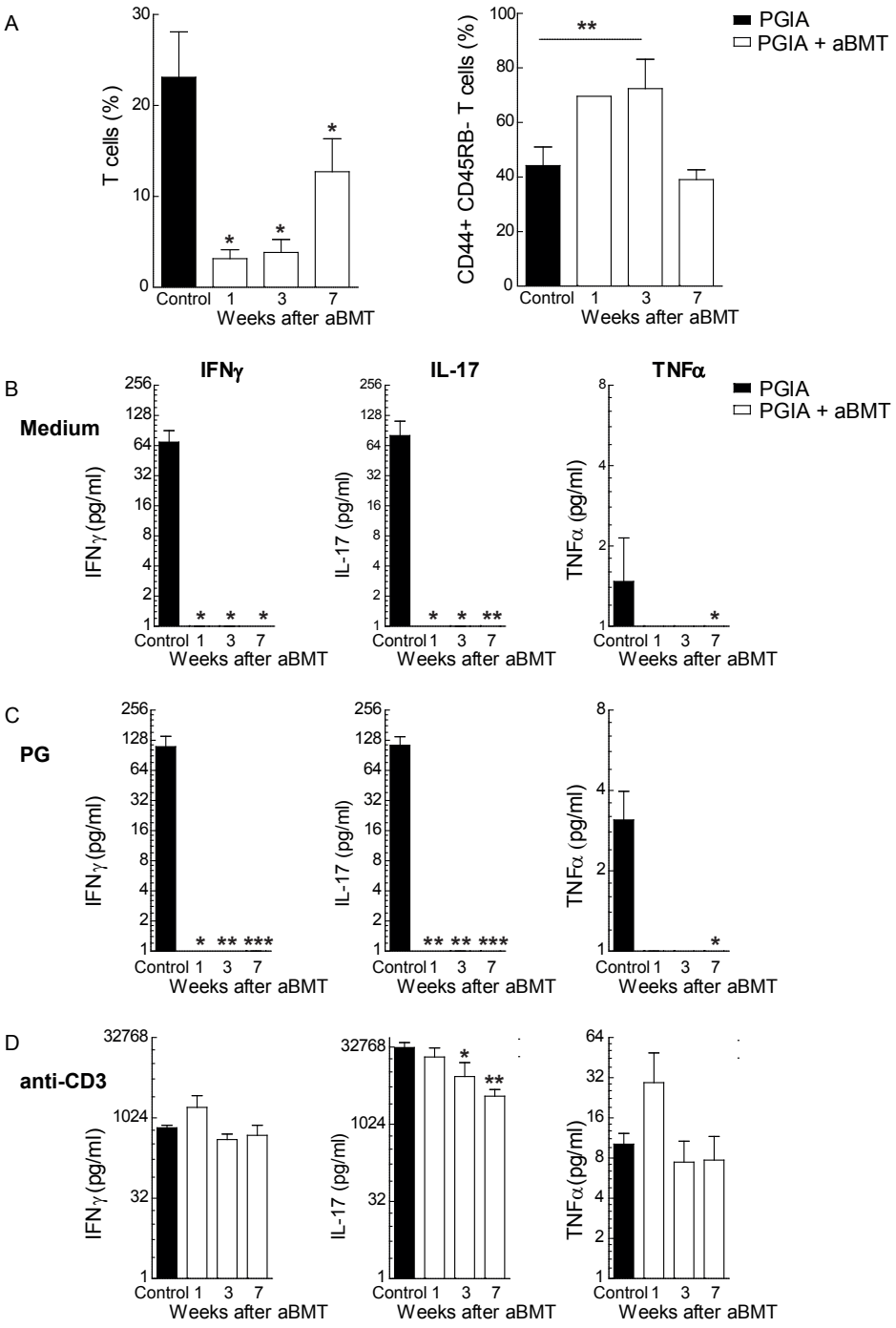


Figure 1. Reduction and stabilization of proteoglycan-induced arthritis (PGIA) after autologous bone marrow transplantation (ABMT). **A,** Left, Arthritis scores after ABMT. Arthritis scores were set to 100%, and the subsequent clinical effect was expressed as a percentage of the score at the time of transplantation. Results are representative of 2 separate experiments. Right, Area under the arthritis score curve. Values are the mean ± SEM (8 PGIA [control], 5 PGIA/T cell–depleted grafts, and 5 PGIA/unmanipulated grafts). * = $P < 0.05$ by Mann-Whitney U test. **B,** T cell proliferation after PG stimulation, as determined by 3H-thymidine incorporation. Values are the mean ± SEM (2 PGIA, 3 PGIA/T cell–depleted grafts, and 4 PGIA/unmanipulated grafts). **C,** Levels of PG-specific IgG1 antibodies after ABMT, as determined by enzyme-linked immunosorbent assay. Values are the mean ± SEM (n = 5–8 PGIA, 4 PGIA/CD4+ T cell–depleted grafts, and 2–5 PGIA/unmanipulated grafts). **D,** Arthritis scores after administration of a booster dose of PG 4–5 weeks following ABMT. Arthritis scores were set to 100%, and values are the percent increase from the day after administration of the boost (n = 3 PGIA and 3 PGIA/unmanipulated grafts). LN = lymph node.


A
T cells (%)
Weeks after aBMT
CD44+ CD45RB- T cells (%)
PGIA
PGIA + aBMT
B
Medium
IFNγ
IL-17
TNFα
IFNγ (pg/ml)
IL-17 (pg/ml)
TNFα (pg/ml)
C
PG
D
anti-CD3
Control
1
3
7

Characteristics of the donor-derived CD4+ T cell compartment and the host CD4+ T cell compartment

As shown in Figure 2, the (antigen-specific) production of proinflammatory cytokines was reduced after ABMT. This could be attributable to a different environment created by conditioning but also by the renewal of the Teff cell population. By using unmanipulated BM with a congenic T cell marker (CD90.1), we were able to investigate the effect of ABMT on changes in the CD4+ T cell compartment. Seven weeks after ABMT, the majority of CD4+ T cells present in spleen and lymph nodes were donor derived, and these donor cells showed increased proliferation compared with the remaining host cells (Figure 3A). Importantly, donor-derived CD4+ T cells were also observed locally, in the synovial fluid of ABMT-treated mice (Figure 3B). As expected, the majority of donor T cells were naive, whereas most host CD4+ T cells showed a memory phenotype (Figure 3C). The percentages of host CD4+ T cells producing IFNγ, IL-17, or TNFα were significantly higher compared with the percentages of donor T cells, in both spleen and lymph nodes, confirming the less-activated status of donor-derived T cells (Figure 3D). Similar results were obtained using CD4+ T cell depleted grafts (results not shown). Taken together, these data demonstrate that ABMT induces renewal of the CD4+ T cell compartment by BM graft–derived T cells that display a more naive and less inflammatory phenotype and also home to the site of inflammation.

Figure 2. Reduced antigen-specific T cell production of proinflammatory cytokines following ABMT. **A**, Left, Percentages of CD4+ T cells following ABMT. Values are the mean ± SEM (control, n = 11; week 1, n = 5; week 3, n = 7; week 7, n = 9). Right, Percentages of memory CD4+ T cells following ABMT. Values are the mean ± SEM (control, n = 11; week 1, n = 1; week 3, n = 5; week 7, n = 6). B–D, T cell–specific cytokine production. Spleen cells were harvested 1, 3, and 7 weeks after ABMT with unmanipulated BM. The cells were cultured in medium alone (B) or in the presence of PG (10 ug/ml) (C) or anti-CD3 (1 ug/ml) (D) for 96 hours. The supernatants were collected and analyzed for interferon-γ (IFNγ), interleukin-17 (IL-17), and tumor necrosis factor α (TNFα) production. Mice with PGIA (control) were killed at different time points, and the data for these mice were pooled (n = 17–19). For mice with PGIA that underwent ABMT, n = 3 at week 1; n = 4 at week 3; and n = 6 at week 7. Values are the mean ± SEM on a log2 scale and are representative of pooled data from 2 separate experiments. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus control, by Mann-Whitney U test. See Figure 1 for other definitions.

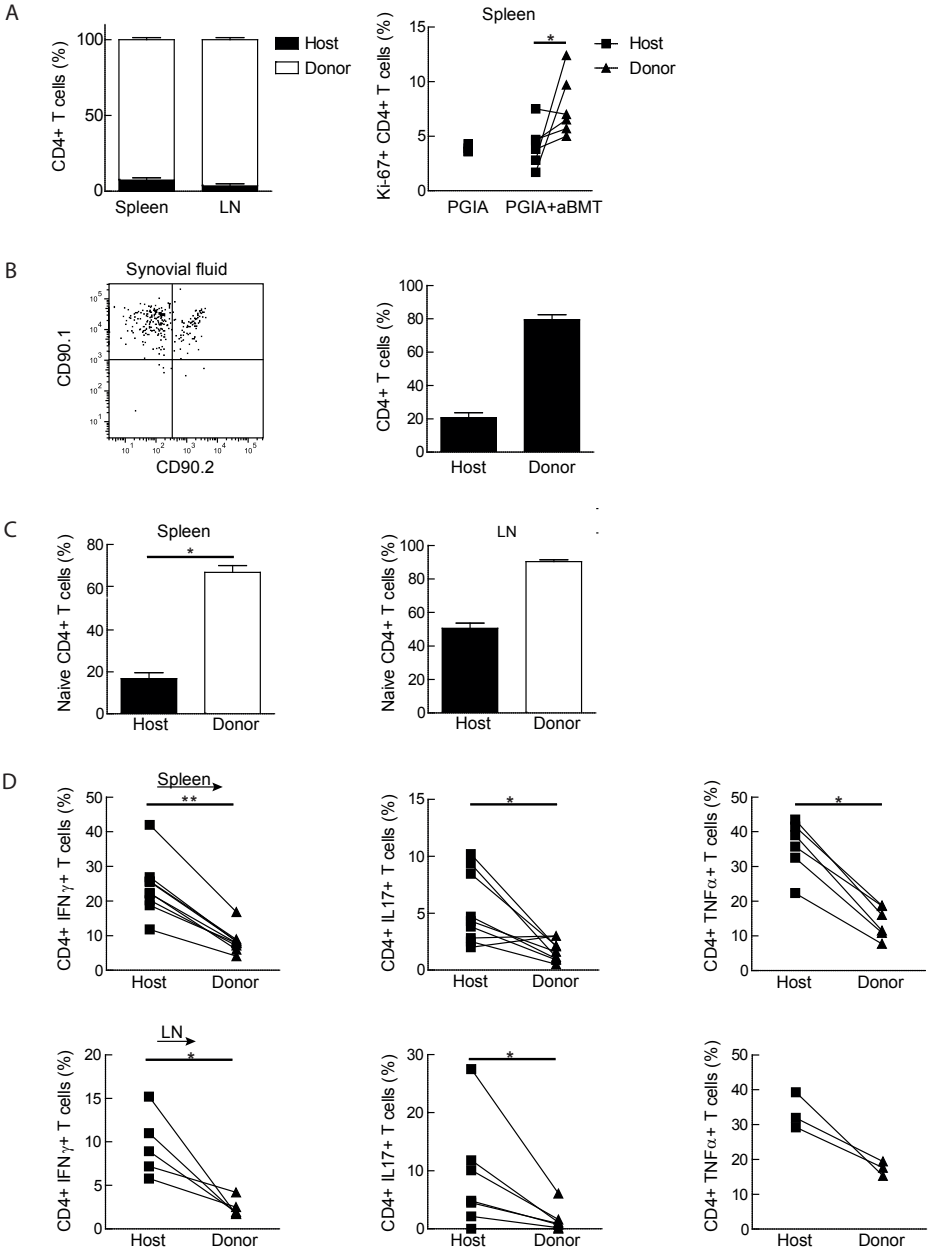


Figure 3. Disease-associated cytokines produced by naive donor-derived CD4+ T cells and residual host-derived T cells. **A,** Left, Percentages of T cell receptor β (TCRβ)+CD4+CD90.2+ (host) cells and TCRβ+CD4+CD90.1+ (donor) cells in spleen and LNs 7 weeks after ABMT (unmanipulated graft). Values are the mean ± SEM (n = 6). Right, Percentages of control, host, and donor Ki-67+TCRβ+CD4+ T cells (control, n = 4; PGIA + ABMT, n = 6). **B,** Left, Fluorescence-activated cell sorting analysis with gating for TCRβ+CD4+ synovial fluid cells. Host T cells are CD90.1CD90.2 double-positive, donor T cells are

CD90.1+. Right, Percentages of host and donor CD4+ T cells in synovial fluid. Values are the mean ± SEM (n = 2). **C,** Percentages of host and donor naive ($CD45RB^{high}CD44^{low}$FoxP3-) CD4+ T cells in spleen (n = 6) and LNs (n = 3). Values are the mean ± SEM (spleen, n = 6; LNs, n = 3). **D,** Intracellular cytokine staining for interferon-γ (IFNγ), interleukin-17 (IL-17), and tumor necrosis factor α (TNFα) in splenocytes (top; n = 6–9) and LN cells (bottom; n = 3–7). Pooled data from 2 separate experiments are shown. * = $P < 0.05$; ** < $P = 0.01$, by Wilcoxon matched pairs signed rank test. See Figure 1 for other definitions.

DISCUSSION

Autologous stem cell transplantation induces stable remission in a substantial portion of patients with severe JIA [1]. Understanding the working mechanisms of ASCT in autoimmune diseases may help in the development of new therapies that result in the same outcome but with less toxic side effects.

One of the original hypotheses for the success of ASCT in autoimmune disease is that ASCT eradicates autoaggressive T cell populations. Although conditioning ablates the T cell repertoire to a large extent, elimination is never complete, and there is a reasonable risk that autoaggressive T cells will persist. In addition, memory T cells will also be infused with the autologous stem cell graft. In peripheral blood stem cell–mobilized grafts, the number of T cells is 10-fold higher than the number in conventional BM grafts, but the T cells have a more naive phenotype [10]. Here, we show in a mouse model of PGIA that the use of unmanipulated BM gives the same clinical results as T cell–depleted BM; this suggests that T cells present in the BM graft have a minimal effect on the clinical course of PGIA following ABMT. Recently, Snowden et al reported that none of the European Group for Blood and Marrow Transplantation registry outcome analyses to date support ex vivo depletion strategies [11]. Consistent with this observation, more intense T cell depletion has also been associated with a higher rate of tolerance failure and the development of secondary autoimmune disease following ASCT [12].

Autoreactive T cells that survive conditioning represent another risk factor for the loss of self-tolerance following ASCT, especially in the setting of autoimmunity. Our results using a PGIA model of syngeneic BMT now demonstrate that remaining host CD4+ T cells proliferate vigorously early after ABMT and display a proinflammatory phenotype characterized by the production of IFNγ, IL-17, and TNFα. Because the development of PGIA is dependent on both IFNγ and IL-17 production [13,14], these results suggest a relatively high risk of loss of tolerance and relapse of arthritis shortly after ABMT. Consistent with this, in our multicenter study of ASCT in patients with JIA, 90% of disease relapses occurred within 9 months after ASCT [4]. However, our data also show that despite the presence of these proinflammatory host CD4+ T cells, basal proinflammatory cytokine production and self antigen–specific cytokine responses are strongly reduced immediately after ABMT. In addition, the risk of early relapses may also be controlled by the presence and expansion

of Treg cells, as shown in a previous study[9]. Taken together, these results show that shortly after ASCT, there is a very delicate balance between lymphopenia-induced immune cell expansion and activation versus immune suppression and immune regulation that will determine the clinical outcome and early relapses.

Following the early reconstitution period, proinflammatory host T cells are thought to be steadily replaced by T cells derived from the autologous graft. In patients with JIA who underwent ASCT, it was suggested that renewal of the T cell compartment induces autoreactive T cells with a more regulatory phenotype[4]. In our mouse model of PGIA, the "donor" cells indeed displayed a more naive and less inflammatory phenotype compared with the "host" cells, despite the enormous expansion of the number of donor cells. Furthermore, autoaggressive T cell responses remained low during the 7-week followup period after BMT. Taken together, our data indicate that ABMT resets the immunologic clock by renewing the functional CD4+ T cell compartment.

Restoration of immune tolerance is still considered the Holy Grail for the treatment of autoimmune diseases. ASCT is the only treatment that can lead to a sustained restoration of the immune balance in patients with severe autoimmune disease. Understanding the immunologic mechanisms of ASCT will help us develop new therapies that have the same clinical outcome but with less toxic side effects.

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PART TWO

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The evolution of biomarkers in rheumatoid arthritis: From clinical research to clinical care

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Background
Treatment efficacy in rheumatoid arthritis (RA) has been measured by clinical response, gross radiological results and some biochemical markers. With the new biologic treatments, markers for disease development, progress, severity and therapy response have evolved.

Objectives
This review focuses on a selection of current markers and the need for better markers for determining RA treatment susceptibility and success.

Methods
A review of the literature was conducted and expert opinions expressed.

Results/conclusion
Current biomarkers mainly focus on disease activity and severity. Biomarkers for treatment response and susceptibility that help clinicians make initial treatment decisions are lacking or insufficient, yet required for optimal control of RA. A combination of biomarkers is necessary to classify a complex immune disease, such as RA.

INTRODUCTION

Rheumatoid arthritis (RA) is the most common systemic autoimmune condition worldwide (1% prevalence) [1]. RA is characterized by persistent inflammation of joint synovium, leading to irreversible joint deformities and functional impairment [2]. While several different pathways of activation of inflammation and tissue damage have been identified, the cause of this autoimmune disease remains undiscovered. Identifying these pathways has aided the development of drugs, which typically try to intervene in the immune or inflammatory response present in RA. Since the beginning of RA drug development, treatment efficacy has been measured by clinical response, gross radiological results and biochemical markers. Currently, biologics are a key step in the evolution of RA treatment. A new era of possible markers for disease development, progress, and severity, and for monitoring therapy response has evolved with the new treatment options. This review focuses on a selection of the current markers and the need for better markers for current RA treatments, especially biomarkers identifying susceptibility to and efficacy of a certain treatment.

Treatment options

Several disease-modifying antirheumatic drugs (DMARDs) are currently available and new drugs are on the horizon (see Table 1). RA is recognized as a complex immune disease, caused by a variety of different pathological processes. Drug treatment that interferes with a single pathological process will probably not result in definitive modulation of the disease. A treatment approach combining several different pathological processes could result in better disease control. Different combinations have been tried [3-5], of which some combinations, such as methotrexate (MTX) with anti-TNF agents, have been shown to have additional benefit when compared with monotherapy[6-8].

A different approach is close monitoring of RA patients, termed tight control, which tailors the treatment strategy to the disease activity of individual RA patients. The TICORA (Tight Control for Rheumatoid Arthritis [9]), FIN-RACo (Finnish, Rheumatoid Arthritis Combination therapy [10,11]), CAMERA (Computer Assisted Management in Early Rheumatoid Arthritis [12]) and the Dutch BeST (Behandel Strategieen [13-15]) studies, among others, have demonstrated the importance of this new concept. Tight control has resulted in greater improvement and a higher percentage of patients meeting the preset aim of low disease activity or remission. This is more effective with early intervention with a biologic agent or combination therapy of a biologic agent and a traditional DMARD than when intervention is started later in the disease course. Combination therapy may even induce remission or allow long-term therapy with MTX to maintain remission [15].

Several drugs and treatment schemes are available or described in the literature but are they really applicable in real world decision-making? For instance, the concept of tight control appears very beneficial in clinical trials but clinical use could be tedious and difficult

Table 1. Selection of current available drugs for RA

Glucocorticoids [123]	Glucocorticoids modulate gene transcription, among other parameters, for example inhibition of transcription of cytokines, adhesion molecules and metalloproteases. They also affect lymphocytes and macrophage activation and neutrophil transport and accelerate breakdown of cytokine mRNA
Disease-modifying anti-rheumatic drugs (traditional) [123]	
Gold salts	Interfere with lymphocyte and monocyte function in vitro and reduce levels of immune complexes. New insights suggest that gold may interfere with the pro-inflammatory transcription factor AP-1, through which IL-1 and TNF-α mediate their effects on collagenase transcription
Methotrexate (MTX)	An antimetabolite that competitively inhibits the enzyme dihydrofolate reductase, which, in cancer cells at least, appears to result in the cell-death of proliferating cells. In RA it may inhibit angiogenesis. The role of the antiproliferative effect of MTX in RA remains unclear
Hydrochloroquine (HCQ)	Antimalarial drugs have a variety of actions, among these is the capacity to interfere with acid-dependent subcellular functions. T and B cells may be inhibited, as well as antigen processing in monocytes and macrophages and IL-1 release of the latter cells may be inhibited
Sulfasalazine (SSZ)	Mechanism of action is unknown but SSZ has effects on T and B cell populations and suppresses levels of IL-1 and TNF-α. It has also been shown to inhibit the transcription factor NF-kB, which is important in lymphocyte proliferation
Leflunomide (LEF)	Prevents DNA synthesis by blocking an enzyme involved in de novo pyrimidine nucleotide biosynthesis. T cells preferentially use this enzyme in pyrimidine synthesis. LEF has been reported to inhibit the production of TNFα, IL-1, reactive oxygen radicals and matrix metalloprotease 3 (MMP-3) by human synovial cells, in vivo and in vitro
Azathioprine (AZA)	Inhibits T and B cell proliferation and activity, immunoglobulin synthesis and IL-2 secretion by inhibiting several steps in the synthesis of purine nucleotides
Disease-modifying anti-rheumatic drugs (biologic)	
Anti-TNF (Etanercept, Infliximab, Adalimumab, Golimumab* [120])	Infliximab, Adalimumab and Golimumab are mAbs that specifically bind TNF. Etanercept is a TNF-receptor Fc-fusion protein that binds TNF and lymphotoxin (LT) family members. By binding TNF, these biologics block the inflammatory characteristics of TNF and so modulate or dampen the inflammation
IL-1 receptor antagonist (IL-1Ra, Anakinra [124])	IL-1 is closely related to inflammation and articular damage in many arthritis models. Anakinra blocks the IL-1 receptor, thus inhibiting the IL-1 activity
Cytotoxic T lymphocyte antigen 4 (CTLA-4)-immunoglobulin fusion protein (Abatacept [125,126])	Blocks the positive co-stimulatory signal between the T cell and antigen-presenting cell (APC) by intervening in the engagement of CD28–CD80/86. CTLA-4 has a higher affinity for CD80/CD86 than for CD28 and functions as a negative regulator of T cell activation
Anti-CD20 (Rituximab, HuMax [125,127])	CD20 is a B-cell-specific surface antigen expressed by all B cells. Proposed contributions of B cells in RA include production of autoantibodies, pro-inflammatory cytokines and antigen/T-cell activation. Rituximab is a chimeric monoclonal antibody that depletes B cells expressing CD20
Anti-IL-6 receptor (anti-IL-6R, Tocilizumab [128,129])*	Levels of IL-6 are directly proportional to levels of CRP and disease severity. Anti-IL-6R blocks IL-6 signaling by inhibiting the binding of IL-6 to its receptor
B Lymphocytes stimulator, BLyS (Belimumab, Atacicept [124])*	Atacicept is a recombinant fusion protein that binds and neutralizes the activity of B lymphocyte stimulator (BLyS, CD257), a key cytokine regulating B cell maturation, proliferation, and survival, and its homolog, a proliferation-inducing ligand (APRIL, CD256)
PEGylated anti-TNF (Certolizumab pegol, CDP870 [130])*	Certolizumab pegol, or CDP870, is a monoclonal humanized anti-TNF-α antibody Fab' fragment linked chemically to polyethylene glycol. It neutralizes both membrane and soluble TNF-α, for which it has a high affinity; it does not bind lymphotoxin
Anti-IL-15 (AMG714, formerly known as HuMax-IL-15 [128,131])*	In patients with established disease the levels of synovial and serum IL-15 correlate strongly to DAS28. AMG714 binds to IL-15 and inhibits IL-15 signaling

*Not yet FDA approved.

since very few rheumatologists apply quantitative measures in clinical decision making [16]. In the case of early and aggressive intervention with biologic agents, promising results are seen but the long-term effects and side effects of many biologic agents are still unknown. In the BeSt study, early and aggressive treatment resulted in an initial improvement. However, after two years the patients in the monotherapy group achieved almost the same improvement in disease activity and functional ability as those in the combination therapy group [17]. Furthermore, a population of RA patients remains untreatable [15] or is on high doses of medication despite all the above-mentioned therapeutic options and schemes.
Because of the variety of new drugs available, the high cost of the new 'biologicals', heterogeneous patient response, and unknown side effects, the need for markers for treatment response and especially treatment susceptibility to assist clinicians in making initial treatment choices is becoming more critical.

Markers for rheumatoid arthritis

The field of biomarker discovery in autoimmune diseases in comparison with other areas, such as cardiovascular disease and cancer, is still far behind. Ideally, the most useful biomarker for an autoimmune disease would be one that reaches abnormal levels (either increased or decreased compared with controls) in conjunction with disease development, that fluctuates in relation to disease severity, and that normalizes following successful treatment [18]. Another and possibly more vital challenge is to identify via biomarkers which patients are susceptible to a specific type of treatment. A wide variety of markers have been documented and can be divided into several categories, such as clinical markers, imaging markers, and a large group of biochemical markers. A selection from each of these groups are reviewed and the clinical relevance discussed below.

Clinical markers/disease activity scores

Potential predictors of disease outcome include clinical (Health Assessment Questionnaire (HAQ), tender joints, swollen joints or pain), environmental, serological (rheumatic factor (RF), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP)), radiographic, and genetic (shared epitope) factors. Many of these factors are combined to achieve disease activity monitoring scores. All these available tools, the DAS 28 or 44 (Disease Activity Score, with 28 or 44 joint-count score), SDAI (Simplified Disease Activity Index), CDAI (Clinical Disease Activity Index), GAS (Global Arthritis Score), RAPID (Routine Assessment of Patient Index Data), and the like, have some degree of predictive value at a group level, as in clinical research trials [19-23]. However, measures of disease activity do not provide adequate information to guide therapy choices of the individual [24]. When looking at the separate parameters of these disease activity scores, several observations can be made. One of the clinical factors, pain, is measured by a visual analog scale, and is known to highly correlate with HAQ and therefore disease activity scores [25]. It is not uncommon that RA

patients have secondary fibromyalgia or other painful conditions unrelated to RA disease activity. Indiscriminate pain assessment can result in over estimating and possibly over-treating the actual disease activity [26].
The DAS28 is rather sensitive for detecting changes in ESR in the (nearly) normal range[27,28]. The relative contribution of tender joints to the DAS28 is much higher than that of swollen joints [26], which are a more specific feature of RA. DAS28 also does not include joints of the feet, which are frequently inflamed in RA [29]. Additionally, subjective variables lead to high inter-observer variation [30,31].
These inaccuracies might explain why fewer than 10% of rheumatologists use questionnaires, such as the HAQ, in routine clinical care, and fewer than 15% perform a formal joint count at each visit [16]. And yet these scoring methodologies are one of the most recognized ways to assess disease activity in the clinical setting. We need new and improved tools to not only assess disease activity but to also assess treatment efficacy and to predict changing disease activity scores in advance, especially if the given treatment slows or stops disease progression.

Imaging markers

Radiography is the traditional gold standard in assessing joint damage in RA patients [32]. It relies on indirect and nonspecific signs for RA, such as joint-space narrowing to indicate cartilage thinning, soft tissue swelling, dislocation and effacement of fat pads, and specific signs, such as bone erosions [33,34]. Several scoring systems are applied in clinical research to quantitate the radiographic data for disease activity and severity, such as the Sharp, van der Heijde, Larsen, and Genant scoring systems and adaptations of the various systems [35]. However, these scores are tedious to handle outside the setting of clinical research. Besides these scoring difficulties, radiography has obvious technical limitations [32]. It cannot predict the disease course or treatment efficacy. In addition, the patient must first suffer typically irreversible structural damage before disease activity presents in the images, thus radiography only shows signs of prior disease activity.
Ultrasound and MRI are more sensitive for assessing erosions and synovitis. MRI bone erosions and bone marrow edema have been demonstrated to be comprised of inflammatory infiltrates in the bone marrow of RA patients [36,37]. This finding suggests that bone marrow edema may be an additional target for therapeutic intervention.
The Outcome Measures in Rheumatoid Arthritis Clinical Trials (OMERACT) adopted an MRI system to evaluate inflammatory and destructive changes in the hands and wrists. This is a semiquantitative assessment system in which erosions, bone marrow edema and synovitis are counted and weighted in a standardized fashion. The study suggested this system to be the standard comparator for a new and alternative MRI method of RA assessment in longitudinal studies [38].

In another study, a modified RA magnetic resonance imaging score (RAMRIS) scoring system for evaluating the wrist (second through fifth metacarpophalangeal joints (MCPs) and metatarsophalangeal joint (MTPs)) was used on 635 RA patients. A highly statistically significant correlation between total RAMRIS scores and disease duration was found. In addition, composite scores of erosions, bone marrow edema, synovitis and bone marrow edema + synovitis correlated significantly with disease duration. All RAMRIS total and composite scores were higher when X-rays showed erosions; but on average, MRIs showed the presence of bone marrow edema and/or synovitis lesions even when X-rays showed no erosion. MRIs proved to be more useful than X-rays for assessing the severity of the erosive damage and inflammatory lesions of synovium and bone. Future research and analyses may result in further insights into how quantitative MRI lesions, especially bone marrow edema and synovitis, rather than erosions might assist in understanding prognosis and help in making treatment decisions [39].

As with MRI, ultrasound is able to detect minimal bone erosions in early target areas and synovial thickening in the peripheral joints affected in RA [32]. Even minimal changes in synovial hypertrophy can be detected more sensitively with ultrasound than with clinical examination [40]. Ultrasound permits excellent assessment of tendon structure and tendon gliding during active and passive motion [32].

Supplementary tools, like contrast-enhanced ultrasound and doppler sonographic vascular imaging, may assess the amount of vascularity in joints and tendons. Vascularity is an early and persistent feature of the inflamed RA synovium and is associated with bone damage [32,41]. However, its distribution and intensity do not directly relate to traditional clinical scores of disease activity [40].

The routine use of contrast agents for ultrasound examinations is not as well established as gadolinium is for MRI [32]. The use and interpretation of ultrasound also requires skilled clinicians. Disadvantages of MRI include higher costs, lower availability, and longer examination time [42], which could be lessened with extremity-MRI. The use of both examinations in standard care also takes some years to be incorporated. Both MRI and ultrasound appear to be good techniques for following disease progression or treatment efficacy by detecting early manifestations before acute damage occurs to bones, tendons or ligaments and could be very useful in making clinical decisions.

Biochemical markers

Several biomarkers have been suggested for predicting outcome and monitoring disease progress in RA. A selection of these biomarkers is listed in Table 2.

Table 2. Selection of biochemical markers

Genetic markers

- HLA-DR4; HLA-DRB-1
- Non-HLA markers 2q34 transition protein 1 (TNP1) and 2q35 (K812, villin 1 (VIL1), desmin (DES)), protein tyrosine phosphatase, non-receptor 22 (PTPN22)
- SNPs: matrix metalloprotease 3 (MMP-3); TNF-receptor-associated factor 1 (TRAF1)/C5; TNF-RII 676; TNF-α-308

Auto-antibodies

- Rheumatoid factor (RF)
- Anticitrullinated protein/peptide antibodies (ACPA):
  - Antikeratin antibodies (AKA)
  - Antifilaggrin antibodies (AFA)
  - Antiperinuclear factor (APF)
  - Anticitrulline antibodies (anti-CCP)
  - Antimutated citrullinated vimentin (MCV)
- Anti-Sa
- Anti-A2/RA33
- Antip68
- Other antinucleair antibodies (e.g., ANA)

Inflammatory markers

- Acute phase reactants
  - Erythrocyte sedimentation rate (ESR)
  - C-Reactive protein (CRP)
  - Acute Serum Amyloid-Associated protein (A-SAA)
- Cytokines/inhibitors (e.g., K-1, TNF-α, IL-6, IL-8, IL-16)
- Calprotectin Myeloid-related protein 8 (MRP 8)
- Thioredoxin

Synovium/cartilage markers

- Hyaluronic acid
- Cartilage oligometric protein (COMP)
- Aggrecan
- Urinary glucosyl-galactosyl-pyridinoline (Glc-Gal-PYD)
- C-terminal crosslinking telopeptide type II (CTX-II)
- Matrix metalloprotease 3 (MMP-3)
- C2C and C1,2C
- CS846-epitope

Bone markers

- Osteocalcin
- Bone sialoprotein (BSP)
- Bone-specific alkaline phosphatase and collagen I polypeptides (PINP)
- C-terminal crosslinking telopeptide type I (CTX-I)
- Receptor activator for nuclear factor κ B ligand (RANKL) and osteoprotegerin ratio

Immunological markers

- Regulatory T cells
- Costimulatory/tolerogenic molecules
- Cytokines/inhibitors (e.g., K-1, TNF-α, IL-6, IL-8, IL-16)

Genetic markers

Several genes have been investigated but human leukocyte antigen (HLA)-DRB1 genes have repeatedly been found to be associated with RA. This association seems particularly true for HLA-DRB1 alleles, which share a similar amino acid sequence known as the shared epitope (SE) [43]. The presence of SE alleles both increases the risk of RA and is associated with more severe disease activity [44,45.] The identification of RA-associated genes outside of the HLA- region resulted in the discovery of protein tyrosine phosphatase, non-receptor 22 (lymphoid) (PTPN22) [46]. This gene has been identified as a major risk factor for several autoimmune diseases. The gene product encoded by PTPN22 increases the tyrosine dephosphorylation of T cell receptor (TCR) proximal signaling molecules, hence a reduction in TCR signaling [47]. These and other genetic markers are however mostly determined in the clinical research setting and add little to the treatment decisions in the clinical setting. Analysis of single nucleotide polymorphisms (SNP) in relevant genes seems promising in associating susceptibility to RA, disease severity and treatment response. Matrix Metalloprotease-3 (MMP-3) is the main metalloprotease secreted by fibroblasts, synovial cells and chondrocytes. It degrades proteglycans, fibronectin, laminin and type IV collagen, and activates other MMPs [48]. An association between radiographic joint destruction and MMP-3 level due to a promoter polymorphism for MMP-3 has been reported but published results are inconsistent [49-51].

Since the biologic agents directed against TNF-α are very effective in treating RA, a prominent role of TNF-α in RA is suspected and therefore much research has been dedicated to this cytokine. Several single nucleotide polymorphisms involved in the pathway of TNF-α have been reported, such as TNF-receptor-associated factor (TRAF)1/C5, TNF-RII 676 and TNF-α-308 [52-54]. The protein encoded by TRAF1 is a member of the TRAF protein family, which associates with and mediates signal transduction from various receptors of the TNF receptor superfamily, including the receptor for TNF-α [52]. The TRAF1/C5 SNP showed an increased susceptibility to and severity of RA, possibly by influencing the structure, function, and/or expression levels of TRAF1 and/or C5 [52]. The association between selected polymorphisms in the TNF-RII and the TNF-α genes and the degree of American College of Rheumatology (ACR) remission criteria, response based on the percentage decrease of symptoms relative to baseline (ACR20 response = 20% decrease etc.) [55] response to anti-TNF-α therapy was investigated in a group of RA patients who all responded to anti-TNF-α therapy (etanercept, infliximab or adalimumab). A significant association between the TNF-RII 676T > G polymorphisms and clinical responsiveness was seen. The patients carrying the wild type (TNF-RII 676TT) genotype had a higher probability of belonging to the ACR70 group in comparison with the TNF-RII 676TG genotype group, which were over represented in the ACR20 group [53]. Other polymorphisms, such as in the promoter gene encoding TNF-α in position-308, showed that patients with the -308G/G genotype were better responders to infliximab than those expressing the A/A

or A/G genotype [54]. Nevertheless, results are still inconsistent. Other mechanisms or a combination of several polymorphisms are probably involved, since the differential response to one anti-TNF-α agent but not necessarily others remains unexplained [56]. The use of SNPs in clinical settings as markers for treatment decisions is currently far from reality but may play a role in a screening process for making these decisions in the future.

Auto-antibodies

Rheumatoid factor (RF) consists of antibodies against the Fc portion of the IgG immunoglobulin and can be detected in 60 – 80% of patients affected with RA [57]. However, RF is non-specific for RA and can be seen in other rheumatic diseases, especially connective tissue disorders, other chronic inflammatory diseases, infections and even in healthy persons [57-60]. The presence of RF is still an important predictor of outcome in RA, and its value is dependent on the stage of the disease [58,59,61]. Early in the course of RA, RF seropositivity is associated with more active disease and the development of bone erosions. Later on, however, seropositivity is less predictive [62,63]. The search for better discriminative markers than RF resulted in diagnostic tests for other auto antibodies directed at citrullinated epitopes carried on proteins/peptides. Within this family of auto antibodies, antiperinuclear factor (APF) and antikeratin antibodies (AKA) are as specific to RA as RF, and like RF are sometimes present before the onset of disease [53,64]. APF and AKA also have been shown to correlate with RA severity and activity [65].

Perhaps even more predictive of disease course is the appearance of anticyclic citrullinated peptides (anti-CCP). Anti-CCP predicts the development of RA and may occur long before the onset of disease symptoms [66,67]. It is associated with severe erosive disease and appears to predict disease progression among RA patients, and may be modulated in patients undergoing biologic treatment [68-72]. Knowing that seropositivity to anti-CCP predicts an aggressive and erosive disease, earlier and aggressive treatment might be warranted.

Overall, auto antibodies are useful markers for predicting the severity of disease progression, as has been done for years with RF. They typically do not make good biomarkers [73], since not every RA patient expresses these factors, and more importantly because they tend to remain detectable even after successful treatment [18]. Anti-CCP might be an exception to that but further research is needed to clarify its role in RA and application to clinical use.

Inflammatory markers

The most frequently used laboratory measures reflecting disease activity are erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) [74]. Both these inflammatory markers are incorporated in several disease activity scoring systems. ESR tends to reflect disease activity of the previous few weeks, whereas CRP reflects more short-term changes in disease activity [75]. Initially ESR was used in the scoring systems but since ESR measurements are easily influenced by confounding factors and CRP might show a better relationship with

disease activity, CRP measurements have gained more support [76]. Factors influencing ESR include age, sex, fibrinogen levels, hypergammaglobulinemia, rheumatoid factor, and anemia [77,78]. Several contrary reports have been published about the use of these markers as predictors of erosive disease. Both markers are used for measuring disease activity but are not specific for RA. Other inflammatory markers, such as the cytokine IL-6, have been suggested as biomarkers, especially as a marker for prognosis. Notably the direct link between cytokine synthesis, particularly TNF-α and IL-6 and CRP, is exemplified in the rapid suppression of IL-6 and CRP following TNF-α blockade [79]. IL-6 is elevated in several other diseases characterized by inflammation, thus it is not RA-specific [80].

A major leukocyte protein that has been shown to correlate well with laboratory and clinical assessments in several inflammatory rheumatic diseases is calprotectin [81,82]. In a recent study, a highly significant correlation was seen with joint damage measures in addition to laboratory and clinical markers of inflammation [83].

Selection of inflammatory markers for assessment are widely determined in the clinic, especially ESR and CRP, and incorporated in clinical decision-making. Many of these acute-phase reactants are non-specific for markers for inflammation, can be influenced by confounding factors, and only show a global view of the current inflammatory process. Even a discrepancy between clinical improvement and changes in acute-phase response during treatment with DMARDs can be seen [84]. The interpretation of these markers must therefore be used with caution in the clinic and should not be used as a solitary instrument for making treatment decisions.

Synovium/cartilage markers

Since markers for inflammation are thought to be nonspecific for disease activity in RA, markers aimed at joint and connective tissue destruction and erosion of articular cartilage may be useful for monitoring RA patients. The concentration of serum biomarkers for cartilage, collagen and synovium for determining breakdown/degeneration have been shown to be related to joint destruction and may be useful in assessing the progression of joint damage. The COmbinatie therapie Bij Reumatoide Artritis (COBRA) trial showed that high baseline levels of C-terminal crosslinked telopeptides of type I and II collagen (CTX-I and CTX-II) were independent predictors of increased risk of radiological progression and accelerated destruction [85]. Thus, detecting RA patients who are at high risk of progressive joint damage very early in the course of the disease. A role for urinary CTX-II in predicting treatment efficacy in patients with RA has been suggested, which is under further investigation [24,86]. Other markers, such as matrix metalloproteases (MMP), are enzymes involved in the degradation of articular cartilage. Several types have been analyzed for significance as a predictor for RA, MMP-3 so far seems to be the most promising predictor for the loss of articular cartilage and total joint damage progression. It is increased in serum of RA and systemic lupus erythematosus (SLE) patients but not in

osteoarthritis patients. Levels of MMP-3 are correlated with inflammatory markers, such as CRP, and reflect disease severity [87,88]. Serum levels of MMP-3 can even decrease following treatment with anti-TNF-α [89,90]. Other markers for cartilage degradation, such as cartilage oligomeric matrix protein (COMP), have been suggested. COMP is a non-collagenous protein component of cartilage that is released as cartilage is degraded and is a marker for early destruction of cartilage [18]. Increased levels are usually detectable before there is radiological evidence of cartilage damage and it is a strong predictor of oncoming aggressive disease [89,91,92]. However, conflicting findings have also been reported on COMP as a predictor of joint damage [93-95]. In summary, although some biological markers of joint metabolism might be significantly increased on a group basis in RA patients, these markers are at present not used as diagnostic tests for RA in individual patients.

Bone markers

Receptor activator for nuclear factor k B ligand (RANKL) and its receptor RANK, both of which belong to the TNF receptor superfamily, are key factors in the stimulation of osteoclast formation and activation [96]. The soluble receptor- like molecule osteoprotegerin (OPG) is a natural inhibitor of RANKL, and the balance between RANKL and OPG regulates bone resorption [97]. In the COBRA trial a low ratio of RANKL/OPG predicted a higher 5-year radiographic progression of joint damage [98]. Other bone turnover markers suggested for use in RA are osteocalcin, bone-specific alkaline phosphatase and collagen I polypeptides (PINP) [63], and bone sialoprotein (BSP) [63,99]. These reflect the overall rate of bone turnover however, which can be affected by a number of conditions (e.g., menopausal status and bone disease such as osteoporosis) [100].

Most of the described markers assess the current activity and severity of the disease or the disease prognosis (future activity and severity). Knowledge of disease severity can support an early and more aggressive treatment approach, thus hopefully preventing or slowing damage due to the inflammatory process. On the other hand this might be at the cost of sustaining more or worse side effects in the short or long-term. Since many of these biomarkers are only used for clinical research and therefore at a group level, not much is known regarding biomarker applicability in a clinical setting on a single patient. Additionally, most of the described biomarkers cannot tell clinicians which treatment options should be used. Biomarkers that predict treatment susceptibility would be extremely useful to clinicians in determining the most effective and appropriate treatment for the patient.

Immunological markers

The introduction of new biologic treatment options for RA via targeted blockade of a specific protein or the removal of a particular cell population provides critical insights into the disease [101]. By patient-focused investigation (bed to bench-side), the opportunity to

understand the immunopathogenesis of the disease and the possibility to find new treatment targets arises. For instance, not all patients respond to any given biologic agent, suggesting that different disease pathways exist in different people. Varying disease pathways may enable classification of disease subtypes based on responsiveness to therapy. These factors may even function as biomarkers for clinical responsiveness. Thus, patient-tailored treatment options could become a reality.

The manipulation of regulatory T (Treg) cells has been a particular focus for the treatment of autoimmune disease and transplantation. Several types of Treg cells have been described. 'Natural', thymically-derived CD4+CD25+ Treg cells, play a role in the maintenance of self tolerance and the prevention of autoimmune disease [102-104]. Treg cells can also be induced and divided into subpopulations based on phenotypic and functional properties, including IL-10- secreting T regulatory 1 (Tr1) cells [105] and T helper 3 (Th3) cells, of which the latter plays a role in mediating tolerance via the production of TGF-β[106]. In RA, these cells might be insufficiently effective, present or even defective [107,108].

The success of treatments with anti-TNF-α agents in RA patients is recognized but not fully understood. Recently it has been shown that effective treatment with Infliximab upregulates the number of peripheral blood Treg (CD4+CD25hi) cells. This however is not due to proliferation of the already available Treg cells but by induction out of a different subset of T cells. These distinct Treg cells express the hallmark marker for Treg functionality, transcription factor forkhead box p3 (Foxp3), and low levels of CD62L, through a TGF-β dependent conversion of CD4+CD25- T cells [107,109]. Others have shown that TNF inhibits the suppressive function of naturally occurring and TGF-β1- induced Treg cells. This inhibition is related to a decrease in Foxp3 mRNA and protein expression by Tregs, by involving signaling through TNF receptor II (TNF-RII). Patients with active RA expressed reduced levels of Foxp3 mRNA and protein and poorly suppressed the proliferation and cytokine secretion of CD4+ effector T cells in vitro. Treatment with Infliximab increased Foxp3 mRNA and protein expression by CD4+CD25hi Tregs and restored their suppressive function [110]. These new insights in the treatment of anti- TNF-α agents show a mechanism for the restoration of tolerance, which should be further explored for new therapeutic strategies and for possible biomarker discovery.

The expression of Foxp3 as a measurement of suppression is however more complex and not as straightforward as it might look. The amount of suppression mediated by Foxp3-positive cells is mainly determined by assays looking at cell frequency and functional characteristics. High cell frequency and/or the suppressive capability of Foxp3-positive cells are seen as if an immunosuppressive environment is encountered [111-115]. However, it has been shown that human effector T cells can transiently upregulate Foxp3 without any regulatory functions and thus increase the Foxp3 cell frequency [116]. Functional studies of Foxp3- expressing T cells use this group of activated effector T cells and are variably contaminated. Even so, these assays might not represent their function seen in vivo, as Treg

cells in in vitro assays are anergic while in vivo they proliferate immensely [117]. CD4+CD25+ Tregs have also been shown to be increased in the inflamed joint and to have a more powerful suppressor activity than peripheral CD4+CD25+ Treg [111-115]. Despite this increased cell frequency and suppressor activity, RA is not suppressed but is very active. An explanation offered by the authors was that the effector cells might be less sensitive for the suppression of CD4+CD25+ Treg [114,115]. The specific field of Treg research in rheumatic diseases is very promising but studies are clearly needed to clarify the role of Tregs in human rheumatic diseases more precisely and to define their potential role as a therapeutic option for the downregulation of inflammation, or even as a biomarker.

When looking at functional instead of phenotypic characteristics of immune cells, as with gene expression analysis, relevant functional characteristics and changes involved in treatment response can be identified. These functional characteristics can than be used as potential biomarkers for response or susceptibility to treatment.

Immune therapy of patients with RA in a Phase II trial employing tolerization to a pro-inflammatory epitope from the heat-shock protein dnaJ (dnaJP1) led to clinical responsiveness and to immune deviation from pro-inflammatory to tolerogenic T cell function. An immune phenotype consisting of the co-expression of some anergic/tolerogenic co-stimulatory molecules, in addition to Foxp3 was found as a prerequisite for responsiveness. Thus, these molecules could possibly function as biomarkers for treatment susceptibility with dnaJP1. The markers described are well known for their immuno-inhibitory function in different disease settings [118]. One could state that patients expressing this tolerogenic immune phenotype before treatment might have a subtype of RA that is less severe and progressive than other subtypes and thus more susceptible to treatment in general. Therefore, these co-stimulatory molecules could prove to be a prognostic biomarker instead of solely a treatment-susceptibility biomarker. In addition, the expression of this immune phenotype might show that early and aggressive treatment is unnecessary in these patients. Further research exploring the underlying mechanistic process of the modulation of the anergic/tolerogenic immune phenotype is necessary. At present they are not of clinical use.

Bed to bench-side research

The understanding of the pathogenesis of RA by studying how specific biologic agents work is an area which is under appreciated but offers a lot of potential. These new agents give rise to more clinical responders but why certain patients respond and others do not is still unknown. The differences in response to the treatments generate an opportunity to dissect the immune system previously only seen in animal models.

We know that anti-TNF agents are superior to conventional DMARDs, which underscores the importance of TNF in RA. The role of TNF is however still unclear in the pathogenesis of RA. For instance, etanercept, infliximab and adalimumab are agents that target TNF

but if one of these agents fails to induce clinical remission, the other agent is still considered as a effective treatment option [119]. Efficacy differences between these agents are documented and could be related to dosing, pharmocokinetics, immunogenicity, ability to block lymphotoxin or fix complement or induction of apoptosis [120]. Elucidating these differences might even assist us in further understanding of the role and pathways of this cytokine in RA.

The same bed to bench-side approach could be used in the scenario of combination therapy. Combination therapy usually results in better clinical response than monotherapy, suggesting that several factors are involved in the disease process and a multiple approach is necessary. The combination of biologic agents is not always recommended, due to an obvious increased risk of immune suppression with ensuing infections, as has been seen with anakinra in combination with etanercept and abatacept with etanercept but further research is necessary [121,122]. The same can be said for biomarker discovery; a single biomarker will not correlate with disease activity but a multi-tiered approach, such as a combination of genomics, proteomics and immunological markers will probably be necessary to classify certain types of RA. It's even more relevant to use a multi-tiered approach when we consider the potential presence of different subsets of RA patients.

Conclusion

The need for biomarkers in RA in this new era of expensive biological treatment options and with the heterogeneity of treatment responses is of increasing importance. Currently many biomarkers are available, which mainly focus on the severity and activity of the disease process. Biomarkers that assist clinicians in making initial treatment choices on the basis of biomarkers for treatment response and susceptibility are lacking or insufficient but are required for optimal control of RA. A combination of several biomarkers will be necessary to classify a complex immune disease, such as RA.

Expert opinion

The swift recent progress in molecular immunology has been translated into the introduction of novel biologic agents that specifically target certain cell phenotypes, cytokines or receptors. The advent of these new therapeutic agents has certainly revolutionized the treatment of RA, leading for the first time to therapeutic regimens that can produce, but not often maintain, remission. The introduction of these new drugs has, however, also created the evident need to identify markers that can help identify not only response to treatment but also susceptibility to it.

As RA is heterogeneous in presentation, prognosis and treatment response among patients, personalized medicine guided via certain (bio)markers might result in a larger group of clinical responders. For example, genetic factors account for 15 – 30% of differences in drug metabolism and response between individuals. However, the diversity of genetic

polymorphisms makes providing definitive results for even a single gene challenging. The specific field of genomics, however, holds very promising aspects in the progress to personalized medicine. Research focusing on genetic alterations in factors that are known to be associated with mechanisms of disease in RA might be the future direction in which to go. For example, study of gene expression in important immune cells in RA or variations in genetic information resulting in a difference of susceptibility to a treatment (as seen with some SNPs in TNF-genes). Many questions still remain, such as which cell population would be of importance, and are difficult to answer. Initially, RA was considered to be solely a T-cell-mediated disease but with the introduction of successful treatments focusing on B cells, the earlier hypothesis does not seem to hold. Even a single nucleotide polymorphism or larger rearrangements might not represent the anticipated clinical outcome. Looking at gene-expression as an outcome measure might help. Which gene expression products are to be targeted and monitored is another question but it is likely to be a combination of several gene expression products providing a scale of likelihood to treatment response. Both the fields of molecular immunology and genomics are highly specialized and mostly used in medical research but are promising diagnostic/predictive tools for future use in the clinic.

The ability to stratify patients on the basis of functional genomics and molecular immunology will eventually lead to a possible optimization of therapeutic regimens, including the ability to extend the proportion of patients who respond to a certain agent by combining, for example, suppression with induction of tolerance. A major effort towards this bedside-to-bench itinerary is ongoing and will probably contribute to bringing the concept of individualized medicine closer to reality.

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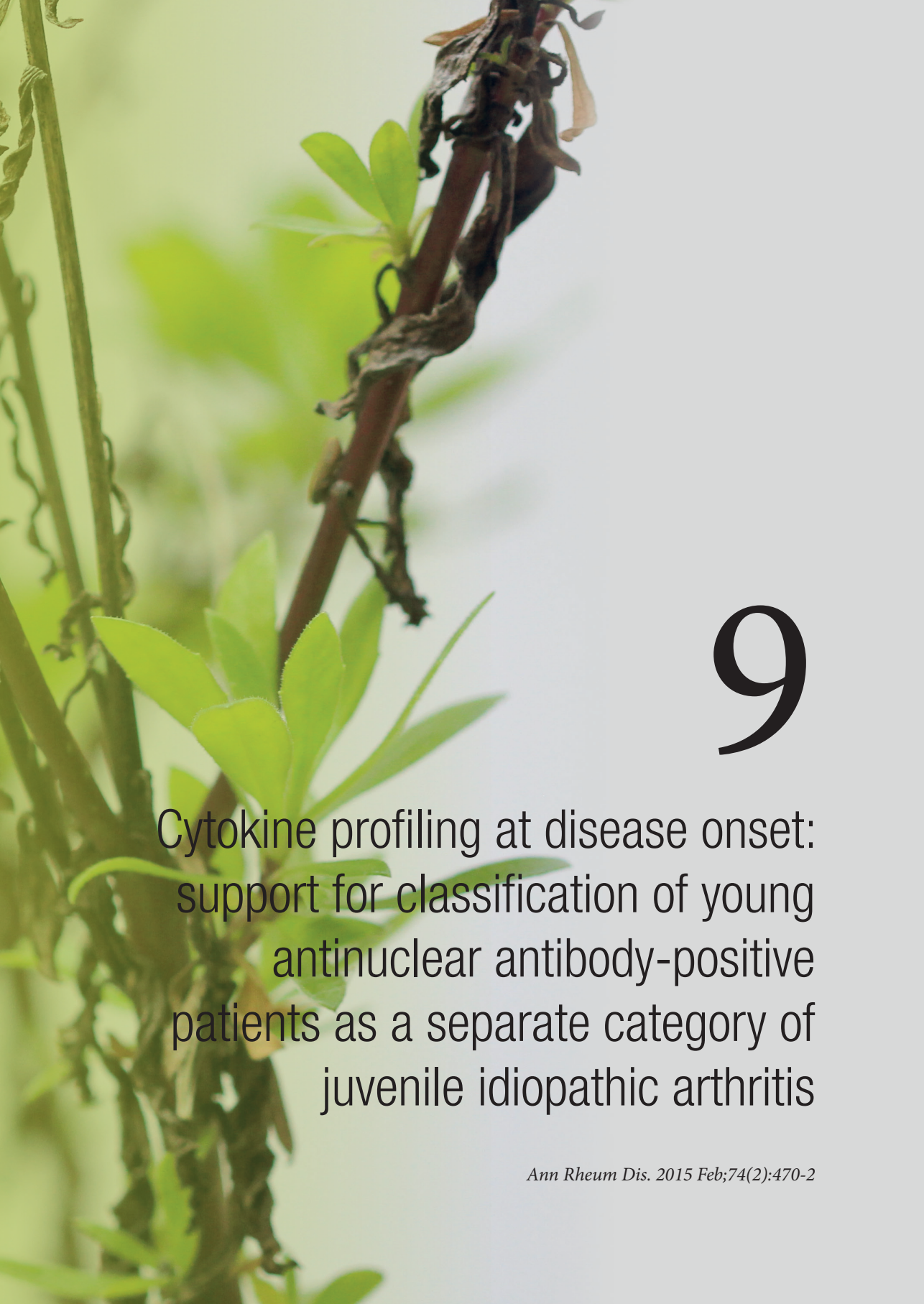

9

Cytokine profiling at disease onset: support for classification of young antinuclear antibody-positive patients as a separate category of juvenile idiopathic arthritis

Ann Rheum Dis. 2015 Feb;74(2):470-2

The current classification for juvenile idiopathic arthritis (JIA) separates the disease entity JIA into seven disease categories based on clinical and laboratory features[1] It is postulated that the combination of early onset of disease and antinuclear anti- body (ANA) positivity better classifies a homogeneous subset of patients than the current International League of Associations for Rheumatology (ILAR) categories, displaying asymmetric arthritis, female predominance and an increased risk of developing iridocyclitis.[2–5] The classification by the number of joints involved might not be an adequate criterion for identifying homogeneous disease entities. We sought out to determine if this homogenous group of young ANA-positive patients could be distinguished from other JIA patients based on their soluble inflammatory profile. These profiles could aid in the differentiation of patient groups by identifying similar underlying inflammatory processes.

This study prospectively followed 40 patients with JIA at onset of disease at their first visit to the paediatric rheumatology clinic for oligoarthritis (n=24) and rheumatoid factor-negative polyarthritis (n=16). Ethics approval was given by the regional review boards. Informed consent was obtained from all patients. The occurrence of iridocylitis was determined by reviewing total clinical history; the time of follow-up did not differ between the groups analysed (table 1). Apart from nonsteroidal anti-inflammatory drugs (NSAID) therapy, no other medications were taken at or before the time of blood sampling and inclusion in the study. Patients were defined as being ANA positive (n=23) if they had at least two positive results at least 3 months apart during follow-up. Proteins were measured in plasma, obtained at onset of disease, by multiplex technology (xMAP, Luminex, Austin, Texas, USA) as described previously.[6, 7] Fifty-seven proteins were analysed and comparisons were considered significant after Bonferroni adjustment setting the significance level from $p<0.05$ to $p<0.000877$ (=0.05/57).

Analysis of various determinants describing the homogenous JIA population such as young age (<6 years), ANA status, sex and development of iridocyclitis separately did not show a distinct cytokine profile after Bonferroni correction. Furthermore, no differences in soluble inflammatory proteins were seen concerning the subtype of JIA. Interestingly, several inflammatory proteins seem to overlap with ANA status and one or more of the other determinants (figure 1A), such as trombopoietin (TPO), C–C motif chemokine 19 (CCL19), soluble IL-2 receptor (sIL-2R) and serum amyloid A1 (SAA1), suggesting a common inflammatory process.

Next, we classified the JIA patients according to both ANA status and age at disease presentation. The group of younger ANA-positive patients have a significantly increased risk (>4 times) for the development of iridocyclitis and displayed increased sIL-2R (median 1608.59 pg/mL vs 408.08 pg/mL, $p<0.001$) and decreased SAA1 (median 3896.90 ng/mL vs 6871.70 ng/mL, $p<0.001$) concentrations (table 1). The differences in sIL-2R and SAA1 were distinct for this group of young ANA-positive patients since other groups of JIA, according to ANA status and age at disease onset, were significantly different (figure 1B).

This study shows that neither ANA status nor the subtype of JIA alone yields a distinct cytokine profile, but that categorising young ANA-positive JIA patients as a separate ILAR subcategory is further supported by low levels of SAA1 and high levels of sIL-2R. Interestingly, blocking of sIL-2R seems to be a valid immunotherapy for some autoimmune uveitis patients.[8] Therefore, it could be that young ANA-positive patients would benefit from such treatment. Validation of these results in a larger cohort is encouraged, especially

Table 1. Clinical characteristics and differentially expressed soluble inflammatory related proteins of young ANA-positive patients compared to a group of ANA-negative and older-ANA-positive patients (Other JIA).

	Young ANA+ JIA(n=11)	**Other JIA (n=29)**	**p-value**
Age, years	1.9 (1.6-3.4)	8.4 (7.4-10.4)	0.000
Gender, female, n (%)	11/11 (100%)	15/29 (52%)	0.004
Iridocylitis	5/11 (45%)	3/29 (10.3%)	0.013
Follow-up, years	7.0 (5.5-7.6)	6.2 (4.7-6.3)	0.254
Inflamed joints	2 (1.1-6.4)	3 (3.2-7.0)	0.385
Soluble proteins:			
IL-1β	11 (13)	2.7 (6.9)	0.041
IL-6	129 (319)	20 (58)	0.013
IL-13	180 (172)	55 (112)	0.023
TSLP	31 (55)	5.6 (16)	0.044
CXCL8	17 (16)	3.9 (6.8)	0.041
HGF	161 (244)	28 (63)	0.015
sICAM-1*	147 (79)	135 (56)	0.044
KIM-1	33 (63)	9.3 (25)	0.028
S100A12*	97 (397)	15.6 (35)	0.030
SAA1§	3.9 (3.3)	6.9 (6.0)	<0.001
sIL-2R*	1.6 (1.3)	0.4 (0.9)	<0.001
Adipsin*	15 (13)	38 (44)	0.028
TPO*	398 (67)	454 (215)	0.017
Adiponectin§	350 (134)	278 (79)	0.035

Age at disease onset, years of follow-up and the number inflamed joints are shown as median (95% CI). Concentrations as pg/mL, or ng/ml (*), or ug/mL (§) and median (IQR) are shown. Independent sample Mann–Whitney U test was used for continuous data, while $\chi 2$ test was used for categorical data. Antihistone antibody measurement was performed by ELISA as recommended by the manufacturer (IBL International, Hamburg, Germany). ANA measurement by indirect immunofluorescence was performed according to the manufacturer's protocol (Euroimmun, Lübeck, Germany). ANA, antinuclear antibody; CXCL8, interleukin 8; HGF, hepatocyte growth factor; IL-1β, interleukin 1beta; IL-6, interleukin 6; IL-13, interleukin 13; JADAS-10, juvenile arthritis disease activity score-10; JIA, juvenile idiopathic arthritis; KIM-1, kidney injury molecule 1; SAA1, serum amyloid A 1; sICAM-1, soluble intracellular adhesion molecule 1; sIL-2R, soluble interleukin 2 receptor; S100A12, Enrage; TPO, thrombopoietin; TSLP, thymic stromal lymphopoietin.

if sIL-2R and SAA1 are to be used as markers of prediction for the development of uveitis. Overall, these results under- score the hypothesis that early onset ANA-positive JIA patients may belong to a separate disease group.

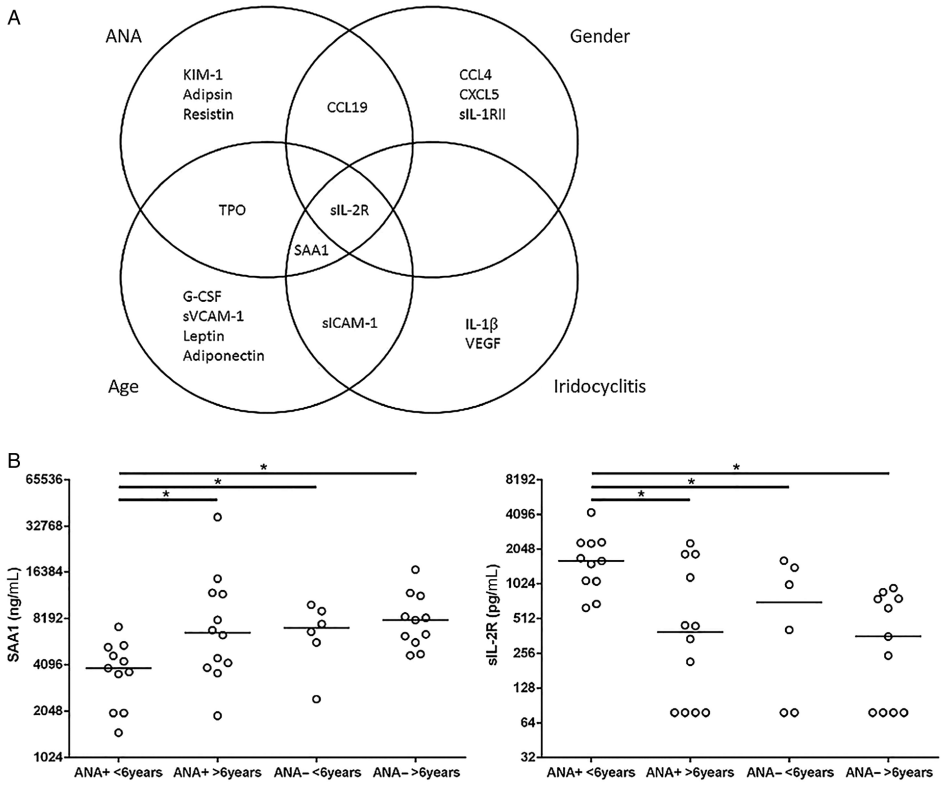

Figure 1. The soluble inflammatory-related markers such as serum amyloid A1 (SAA1) and soluble IL-2 receptor (sIL-2R) are associated with young age at disease onset and a positive antinuclear antibody (ANA) status in juvenile idiopathic arthritis patients. (A) Venn diagram of ANA status, gender, age at disease onset (6 years and younger vs older than 6 years) and development of iridocyclitis. Venn diagram illustrating that C–C motif chemokine 19 (CCL19) is associated with both ANA status and gender, while trombopoietin (TPO) is associated with ANA status and age at disease onset. SAA1 is associated with ANA status, age at disease onset and the risk of developing iridocyclitis and sIL-2R with all the determinants, suggesting that a common inflammatory process is probable. Cytokines shown are $p<0.05$ per group. (B) Dotplots of SAA1 (left) and sIL-2R (right) concentrations according to ANA status and age at disease onset. The concentrations of SAA1 and sIL-2R are differentially expressed in the group of young ANA-positive patients in comparison with the other groups. Independent sample Mann–Whitney U test was used. Median shown, *$p<0.05$. CCL4, chemokine (C-C motif) ligand 4; CXCL5, chemokine (C-X-C motif) ligand 5; G-CSF, granulocyte-colony stimulating factor; IL-1β, interleukin 1beta; KIM-1, kidney injury molecule 1; sICAM-1, soluble intracellular adhesion molecule-1; sIL-1RII, soluble interleukin-1 receptor type II; sVCAM-1, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

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10

General discussion

PART ONE

The importance of understanding the dynamics of T cell homeostasis

Throughout life, in absence of disease, the size and composition of the peripheral T lymphocyte pool is tightly regulated and maintained at relatively constant levels. [1, 2] This T cell homeostasis depends on two sources: thymopoiesis and homeostatic proliferation. The thymus is responsible for the production of new naïve T cells, but thymic involution starts already after the first year of life. [3-5] T-cell homeostasis is dependent on many factors and seems to differ between different types of T cells (figure 1). Peripheral expansion plays a important role in human T cell homeostasis and has been mostly recognized in memory T cells. [6] For naïve T cells, interleukin-7 (IL-7) (among others) in conjunction with low-affinity interactions with (self-)peptide/MHC complexes are needed to maintain homeostasis. [7-10] Under lymphoreplete conditions, these low-affinity self-peptide/MHC interactions induce "survival signals" to naïve T cells but do not induce division. On the other hand, in the case of lymphopenia these same signals stimulate naïve T-cell division. [6, 11, 12] In addition, expansion of T cells via peripheral proliferation and even thymopoiesis can be regulated by the suppressive capability of regulatory T cells (Treg). [13, 14]

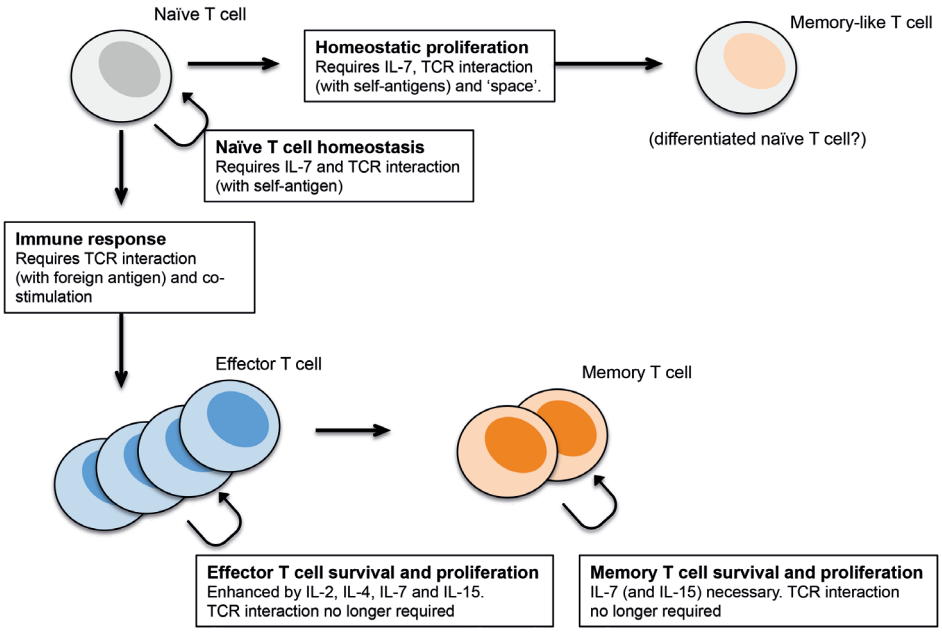

Figure 1. Schematic representation of T cell subset homeostasis

TCR, T-cell receptor; IL-7, Interleukin-7. Figure adapted from Nature Reviews Immunology 2, 547-556 (August 2002)

Many questions still remain unanswered regarding homeostasis of T cells and T cell subsets in both health and disease, for instance; How important is the role of thymopoiesis in T cell homeostasis of neonates? Are naïve T cells really as naïve as their name claims after homeostatic proliferation? How does autologous stem cell transplantation and the following T cell reconstitution contribute to clinical cure? Are T cell lymphopenia and development of autoimmune disease related in immune competent indivuals?
These and others questions were the driving factor of the research presented in this thesis and are described and discussed below.

What can we learn from T cell reconstitution after (autologous) stem cell transplantation?

Autologous stem cell transplantation is used as a last resort treatment for several diseases such as autoimmune diseases. [15-18] Patients that are facing severely debilitation or imminent death after frequent therapy failure are primarily selected for transplantation. Further expansion of the patients who might be eligible for transplantation is restricted due to the toxicity of the treatment, restrictions from regulatory agencies and the uncertainty about how this treatment is exactly rewiring a faulty immune system.[19] However, (autologous) stem cell transplantation has shown to be the only treatment able to induce a long-term drug- and symptom-free remission in several autoimmune diseases. [15, 20-23] Further understanding of this curative potential without the high toxicity is needed to expand patient eligibility and optimize current treatment regimes. It's however not clear which cells must be destroyed prior to transplant. Nor is it known which immune mechanism keeps disease at bay after the stem cell transplantation.

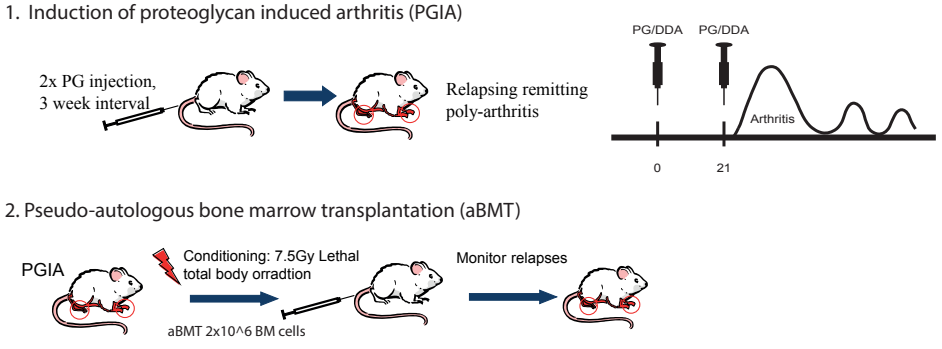

Figure 2. Schematic representation of the proteoglycan-induced arthritis model (1) and the autologous bone marrow transplantation model. BM, bone marrow; Gy, gray; DDA, dimethyldioctadecyl-ammonium bromide.

We utilized an experimental mouse model of autologous (syngeneic) bone marrow transplantation to determine the reconstitution dynamics of both host and donor lymphocytes (figure 2). With the use of congenic markers it is possible to distinguish donor and host lymphocytes in the experimental autologous setting, which is not possible in the human setting. Human clinical samples before and after autologous transplantation were also assessed adjacent to experimental bone marrow transplantation and are described in chapter 6 and 7.

Early after transplantation the radiation surviving T cells from the host expand, followed by thymic-derived (donor) T cells that in the end outcompete the host T cells. This is the case for both effector T cells as regulatory T cells (Treg). Restimulation with the disease-inciting antigen did not result in an increased pro-inflammatory response post-transplantation, in contrary to the response observed in non-transplanted diseased mice. This indicates a renewal of the (thymic-derived) T cell compartment. With thymic-derived T cells a renewal and broadening of the TCR Vbeta repertoire of CD4+ T cells was apparent. Regulatory T cells are thought to be important players for disease remission in autologous hematopoietic stem cell transplantation (aHSCT) treated AID, especially in the lymphopenic reconstitution phase when the delicate immune balance has to be re-established.[24, 25] The contribution of the donor or host Treg compartment in disease remission was however unknown. In the setting of human autoimmune disease, we showed that Treg had a remarkably oligoclonocal TCR Vbeta repertoire, especially in comparison to non-Treg, prior to aHSCT[1]. This repertoire however broadened after aHSCT and corresponded to clinical response. One patient did not display expansion of the Treg TCR Vbeta repertoire and showed disease relapse after stem cell transplantation concordantly. Further studies indicated that Treg played an important role in the clinical response and suppression of autoimmune diseases. It is unknown why the Treg TCR Vbeta repertoire was narrower than non-Treg during autoimmune disease and if the measured repertoire was directed towards disease specific antigens. It is important to take into account that the blood T cell compartment only represents a very small percentage (<3%) of the total T cell population and does not have to be representative of T cells at the site of inflammation, for instance the joint in autoimmune arthritis.[26] In comparison to the circulation, an increased proportion of Treg is present within the inflamed joint [27-30], but with an unknown TCR diversity. With autoimmune disease, expansion of available self-antigens is to be expected due to collateral damage of inflammation and involves antigens not involved in the primary response. It could therefore be that an increased diversity of Treg over non-Treg is needed to control expansion of autoreactivity, at the site of inflammation. In addition, while the TCR sequences did not overlap between Treg and non-Treg, this does not necessarily

[1] Autologous bone marrow transplantation (aBMT) was used for the murine model, while autologous hematopoietic stem cell transplantation (aHSCT) for the human setting

indicate that they also recognized different antigens, as a TCR can potentially recognize more than 1 specific antigen. Taking into account the local inflammation and unknown antigen specificity of the TCRs present, it is difficult to determine the role of these specific TCR Vbeta measured in the circulation of autoimmune disease. Clearly this remarkable finding warrants further investigation in different disease settings and at the site of inflammation.

While the T cell compartment following aBMT was predominantly graft-derived, the majority of the mice did however still had some minor inflammation of the joints. The recurrence of disease is also seen in the human transplantation setting, and can be up to several years after transplantation. [15, 31, 32] Host-derived T cells are the first to expand after lymphodepletion (lymphopenia-induced proliferation) [33] and it is believed that this expansion might be responsible for the persistence of inflammation, due to their autoreactive potential. We therefore initiated a therapeutic approach by infusing Treg with the graft to suppress early reconstitution of these (autoreactive) conditioning surviving T-cells. Treg infusion did not result in any clinical benefit; even a trend towards increased disease severity was noted. Non-Treg T cell reconstitution was also altered after Treg therapy, and was especially delayed in the donor compartment. So in contrary to the allogeneic setting when Treg infusion shows clinical benefit by suppressing graft versus host disease (GvHD) [34, 35], it does not seem to be clinical beneficial in the autologous setting for autoimmunity, at least in the model we used. Timing of the Treg infusion and the use of repetitive infusions could of course be adjusted or implemented to optimize their suppressive potential, but it is expected that only general suppression would be accomplished in the end. In addition, it might even affect the donor compartment in greater degree as residual host cells undergoing homeostatic proliferation seem to be resistant to costimulatory blockade, and remain so even after proliferation has ceased. [36] Recent evidence also suggests that thymic Treg cell development is controlled by a negative feedback loop in which mature peripheral progeny cells return to the thymus and restrain development of precursors of Treg cells. [14] Hereby they may further delay the reconstitution of the donor compartment and thus potentially influence the clinical response. Instead of general suppression induced by total Treg infusion, a possibility would be to infuse Treg cells specific for the disease-inciting antigen. For now it is unknown what the disease-inciting antigen is in humans. Recently, the use of regulatory T cells that recognize an ubiquitous stress-inducible self-antigen, involved in the perpetuation of inflammation, have shown beneficial results in dampening inflammation. [37] Another strategy would be to specifically suppress or delete the conditioning surviving (host) auto-reactive cells, but that is for now unfortunately not yet possible.

So what did we learn from T cell reconstitution after autologous hematopoietic stem cell transplantation (aHSCT)? First of all, the donor and host T cell compartment reconstitution differs and primarily depended on peripheral proliferation or thymic output respectively.

The host T cell compartment initially expanded after aHSCT, followed by the thymic-derived donor compartment. Expansion of the TCR Vbeta repertoire post-HSCT was especially important in the Treg population, as persistence of an oligoclonal TCR Vbeta repertoire seemed to result in clinical disease relapse. In addition, therapy failure could also be dependent on the amount of self-reactive (host) T cells that had reconstituted after lymphoablation. Treg therapy did not seem to add any clinical benefit to the autologous transplantation setting and might even harm reconstitution.
While host cells that survive conditioning now seem to be unwanted due to their auto-reactive potential, reconstitution of some host cells might actually be beneficial post-transplantation. In the early post-transplantation period viral reactivation and invading pathogens are of serious risk to the patient without an adequate immune response. [38-40] Further understanding of the reconstitution dynamics of both host and donor cells in both circulation and site of inflammation is needed to identify potential targets for intervention in harmful and helpful reconstitution.

Neonatal thymectomy, an interesting model for premature immune aging and homeostatic proliferation

Premature immune aging and functional differentiation in the naïve T cell compartment

In the early 1960s, the essential role of the thymus in the immune system was discovered.[41, 42] Since that time many scientist have tried to find a way to identify recent thymic emigrants and distill their function. Expression of CD31[43] or PTK7 [44] and TREC (thymic recent emigrants circles) [45, 46] contents in naïve T cells have made it possible to identify the amount of recent thymic emigrants (RTE) in the circulation. While RTE, as well as naïve T cells, have by some been designated as T cells with a reduced immune function, Gibbons et al have recently shown that IL-8 is highly expressed in naïve T cells of newborns and could be potentially involved in a protective mechanism against bacterial infection in newborns. [47] Showing that naïve T cells have a distinct effector function besides skewing to an effector/memory T cell subset after antigen recognition. In chapter 3, we further explored the function and phenotype of naïve T cells in neonatally thymectomized children. We showed that after neonatal thymectomy the ability of naïve CD4+ T cells, specifically CD31+ naïve CD4+ T cells to produce IL-8 was greatly diminished. In combination with a lowered and delayed calcium flux upon stimulation, this suggested that the naïve CD4+ T cell compartment was functionally altered after removal of the thymus. With healthy immune aging, naïve T cells also show a lower production of IL-8 and lower overall calcium flux upon stimulation [47, 48], indicating 'aging' in the most naïve T cell compartment while preserving their naïve phenotype. This is further supported by the fact that CD31+ naïve CD4+ T cells of thymectomized children had a similar transcriptional profile to CD31- naïve CD4+ T cells of the same patient, while both naïve subsets greatly differed from their healthy control counterpart. Despite the expression of CD31, these cells resembled naïve

T cells that have a proliferative history, indicating partial differentiation within the naïve T cell compartment. Production of IFNγ, usually seen in differentiated effector T cells, was also present in naïve T cells after neonatal thymectomy. The concept of partial differentiation of naïve T cells was supported by signaling alterations seen in older T cells. These older naïve T cells show reduced miR-181a expression that is at least in part the cause of lower calcium flux in naïve T cells with age. [49] In addition, recently an increase of naïve T cells expressing CD25dim has been described in aged individuals, likely caused by homeostatic proliferation. [50] After thymectomy, we observed a decrease in CD25- naïve T cells, and an increase in memory T cells irrespective of CD25 expression, but no difference was detected for naïve T cells expressing CD25dim or CD25int (data not shown). While downregulation of IL-8 in CD31+ naïve T cells after neonatal thymectomy was at least in part cytokine-mediated, the induction of CD25 expression seemed to be (low-affinity) TCR activation mediated with partial loss of CD31, suggesting a dissimilar level of differentiation.

The precise role of IL-8 production by RTE is uncertain. IL-8 is a well-known chemokine involved in neutrophil chemotaxis [51, 52] and the activation of γδ T cells [47], among others. The majority of plasma IL-8 is however produced by various other cell types [53] and not RTE. This is in support with the unaltered plasma concentration of IL-8 after neonatal thymectomy (data not shown). With the fact that IL-8 production by RTE is rapidly decreased after cell division by cytokine stimulation a role for systemic infection control of IL-8 by RTE is less likely. This does not exclude the potential of a potent local effect in the initiation of the adaptive response, as naïve T cells are at the initial critical steps of the adaptive immune response. The capability of naïve T cells to produce IL-8 might be reflective of RTE. After aHSCT, an initial increase of IL-8 producing naïve T cells was seen early post-transplantation, fitting increased thymic export, followed by a decline in the following period (years) of peripheral proliferation (preliminary data in collaboration with E.M. Delemarre).

Later in life, thymic tissue regenerated in the majority of patients, resulting in functional restoration of the naïve T cell calcium flux and IL-8 production upon stimulation. Absence of thymic regeneration resulted in the persistence of low recent thymic emigrants and naïve T cells, with diminished calcium flux and IL-8 production. Implying an important role for the thymus in the regulation of a functional naïve T cell compartment.

Overall, neonatal thymectomy resulted in premature immune aging [54, 55], in part by partial differentiation of the naïve T cell compartment. Thymic export was essential for maintenance of a functional naïve T cell compartment, as thymic tissue regeneration resulted in rejuvenation of the naïve T cell compartment, summarized in figure 3. Further investigation of IL-8 production by RTE and its clinical relevance is needed and might be utilized as functional biomarker of RTE.

Induction of autoreactivity by homeostatic proliferation after neonatal thymectomy

Besides alterations in the naïve T cell compartment, neonatal thymectomy predominantly resulted in the skewing towards a memory phenotype with the loss of naïve T cells. [55-58] As described earlier, this expansion was dependent on homeostatic cytokines as well as (self-)antigen recognition. The role of lymphopenia-induced homeostatic proliferation (LIP) in the induction of autoimmune disease has been studied in several autoimmune prone mouse models [59-61], and only recently in humans who had multiple sclerosis. [62] Recent human data supports that autoimmune development after lymphocyte depletion is caused by LIP. [62, 63] However, in both murine and human studies these conclusions are mainly based in settings of pre-existing or increased susceptibility of auto-reactivity. In contrast to what is seen in multiple sclerosis patients [62], no autoimmune disease was detected up to 29 years after neonatal thymectomy despite increased auto-reactivity. Differences in IL-21 genotype [63], the severity or duration of lymphopenia, and increased proliferation of Treg after neonatal thymectomy [64] could contribute to the lack of autoimmune disease in thymectomized patients in comparison to multiple sclerosis patients.

Neonatally thymectomized children that had the highest proportion of memory T cells, most expanded, had detectable autoantibodies. Prior samples of two children were available, 11 and 37 months, and did not reveal any autoantibodies, but a high percentage of memory T cells were already apparent. This might suggest that T cell expansion precedes the development of autoantibody development. Assessment of the autoantibody profile in thymectomized children was characterized by a lower reactivity of some IgM autoantibodies and greater reactivity to some IgG autoantibodies, in comparison to age-matched healthy controls. This might indicate that in the early years post-thymectomy maintenance of self-tolerance was disturbed, as IgG autoantibodies have been associated with autoimmune disease, while IgM autoantibodies have been associated with maintenance of self-tolerance.[65, 66]

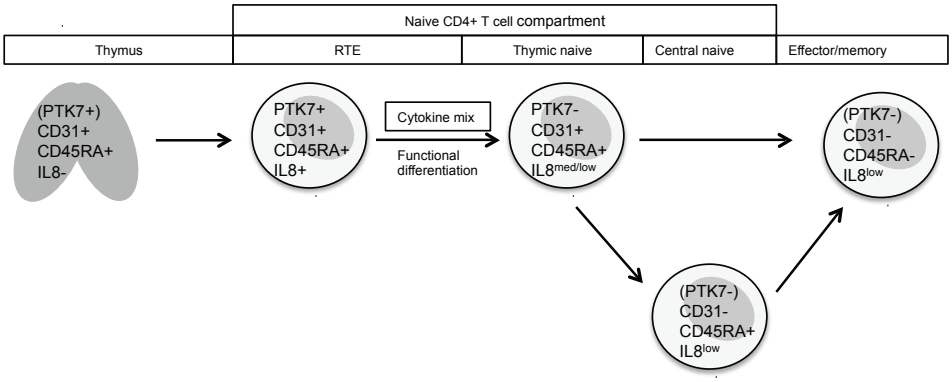

Figure 3. Schematic depiction of naïve T cell homeostasis and differentiation.

No noticeable changes were detected in the proportion of different B cell types, even those associated with auto-reactivity. It therefore seems that the altered antibody profile is primarily due to an altered reactivity of the B cell response and directed to self. Despite this increase in autoantibodies, even up to 29 years of age, no autoimmune disease was evident. Concomitant expansion of the regulatory T cell compartment during these crucial first years after neonatal thymectomy might have played a role in the regulation of excessive autoreactivity from becoming clinical overt.

A decade or more after neonatal thymectomy a large proportion of children showed auto-reactivity focused on antinuclear antigens (ANA). It is unknown why the reactivity was predominantly present towards ANA. In murine models, timing of thymectomy has important consequences on the type of auto-reactivity in murine models. Thymectomy at day 3 results in autoimmune gastritis [67, 68], while at 3 weeks in diabetes mellitus. [69, 70] In the studied cohort, all neonates were thymectomized within their first month of life. However, therapeutic thymectomy for myasthenia gravis in adults has also been shown to induce ANA positive autoimmune disease [71, 72], suggesting that reactivity towards ANA is not predominantly timing dependent.

The increased autoreactivity however cannot be solely the consequences of the removal of the thymus and the compensatory peripheral proliferation, as all these children also had major surgery within their first month of life. While the development of autoantibodies due to surgery is not extensively studied and mostly focused to specific tissues, such as anti-heart antibodies, cardiac surgery has been associated with appearance of autoantibodies. [73 74 75] However, these responses are usually transient and do not always correlate with the amount of circulating corresponding antigens. After neonatal thymectomy the generation of autoantibodies was associated with T cell expansion and were detected even up to 29 years after neonatal thymectomy. Together this suggests that the long-lasting altered autoantibody response was mainly the consequence of the absence of a thymus and subsequent HP in the years after surgery but it may have been fueled by acute trauma during surgery. In several patients without thymic regeneration, no auto-reactivity towards ANA or ANCA was detected, while there was still increased proliferation and a predominantly memory skewed T cell compartment. It could be that auto-reactivity is present but (i) not towards ANA or ANCA (ii), auto-reactivity was better controlled, or (iii) HP was not focused towards self-antigens in these patients. An additional group of children that received similar surgery but without thymectomy would be needed to further distill the role of the thymus.

It is unknown why some patients did not regenerate any thymic tissue while others did. A difference in soluble factors, such as IL-7, might be present that induced thymic regeneration. IL-7 therapy is believed as an important cytokine in immune rejuvenation and has been used as a moderately successful therapy to increase naïve T cells. [76] While increased plasma IL-7 is documented after neonatal thymectomy [58], we only saw a relative

increase of IL-7 per cell, including children who did not regenerate thymic tissue (data not shown). While we used two different cohorts of thymectomized children in this study (1-5 years and >10 years of age), a longitudinal follow-up is needed of patients from the time of thymectomy till adolescence to assess potential triggers of thymic regeneration or lack of it. Cytokine therapy, such as IL-7 therapy, might indeed result in an increase of naive T cells. However, it might also potentially result in the loss of RTE associated function within this compartment, as shown in chapter 3. It is for now unknown what the clinical consequences are of this alteration.

Overall, the thymus plays an important role in the phenotypical and functional maintenance of naïve T cells, as summarized in figure 1&3. Functional thymic regeneration occured in the majority of children. While many factors can be involved in the regeneration of thymic tissue it is very likely that lack of thymic regeneration was due to thorough surgical removal resulting in the absence of a platform for thymic regeneration. Partial removal of the thymus does not seem to result in an evident T cell lymphopenia [77] with consequent T cell expansion and should therefor be advocated, as it is for now unknown if described alterations in the autoantibody profile of total thymectomized children will ever or prematurely develop into clinical autoimmune diseased later in life.

Key messages

- Autologous bone marrow transplantation results in renewal of the T cell and Treg compartment, via thymic-derived donor T cell expansion.
- TCR Vbeta repertoire of Treg cells is narrower than non-Treg in autoimmune disease, but broadens after autologous stem cell transplantation and seems correlated to clinical outcome.
- The naïve T cell compartment is not truly naïve and can be differentiated by phenotype and function
- IL-8 production by naïve T cells is enriched in PTK7+ recent thymic emigrants, but lost after cytokine stimulation.
- Neonatal thymectomy results in the increased development of autoreactivity, but not in autoimmune disease (for now), possibly due to preferential Treg expansion post-thymectomy.

This thesis illustrates the dynamics of the T cell compartment to reconstitute in the lymphopenic setting including the temporary and permanent consequences of this process. Hence, the immune system forgives but does not forget.

Of further interest

This thesis describes several new conceptual advances in understanding T cell reconstitution and homeostasis that can be potentially beneficial for the young and elderly. All human data described here have been gathered predominantly from children. Research gathered from adult subjects cannot be simply translated to the pediatric setting, as is apparent from the differences shown in naïve T cell homeostasis.

Pediatric research is hampered by several factors, such as ethical considerations, amount of material that can be obtained, techniques/protocols that can utilize the small amount of material obtained, and lack of background information in children to assess risk or benefit. In line with the limited sample amount that can be obtained in children, we developed a new protocol for FOXP3 epigenetic analysis that opens the possibility for analysis in children and neonates, chapter 4. Which will help to shed new light on the development and stability of Treg in children. Further research in children is necessary as many other biological processes are different from adults and need to be addressed.

The focus of this thesis was primarily on homeostasis via T cell reconstitution from triggers within the human body. This tolerance to self is a continuous process, and has to be maintained while enduring numerous triggers from outside and inside of the body. One of these 'outside' triggers are microorganisms, which can be damaging and cause infection, while others we tolerate. We harbor more microbial cells and viruses in and on us than human cells [78] and they play a major role in health and disease, sometimes referred to as our "forgotten organ". [79] While we have to endure these triggers during T cell homeostasis they also play an important role in this process, as intestinal microbiota are required for spontaneous murine T cell proliferation. Adoptively transferred polyclonal $CD4^+$ T cells do not undergo spontaneous proliferation in germfree SCID (severe combined immunodeficiency) mice but do in conventionally reared SCID mice. [80] Depletion of intestinal microbiota resulted in significant reduction of spontaneous and in lymphopenia-induced ANA production and autoimmune disease.[81] In particular, increased colonization with segmented filamentous bacteria (SFB) influenced ANA production.[82, 83] In contrast, commensal bacteria belonging to the *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Lactobacillus* genera are associated with inflammatory/autoimmune disease protection.[84] However, the relationship between gut microbiota, immunity, and disease is very complex. For instance, SFB are protective in type 1 diabetes, but causes disease in mouse models of EAE or arthritis.[84]

The human microbial diversity increases and converges toward an adult-like microbiota by the end of the first 3–5 years of life.[85] Once established, the composition of the gut microbiota is relatively stable throughout adult life, but can be altered as a result of bacterial infections, antibiotic treatment, lifestyle, surgery, and a long-term change in diet. [85] Overall, with the understanding of the impact of specific commensal bacteria on the immune response, and the understanding of the genetic and environmental factors that shape the

composition of the microbiota, it should be possible to design personalized therapies capable of modulating T cell homeostasis. This could for instance be in the form of personalized probiotics, antibiotics and microbial transplantation. The interplay between microbiota and immunity is an interesting field of study and of great importance in further understanding infection and immunity.

PART TWO

Immunological biomarkers for distinct groups of autoimmune disease

Autoimmune diseases are categorized in several different groups depending on their clinical features and target of auto-reactivity. For juvenile idiopathic arthritis, seven different categories have been described that classify the disease according to clinical and laboratory findings.[86] Interestingly, in this classification children are split in a different category if they have 5 or more joints involved (poly-articular JIA, pJIA) or less than 5 (oligoarticular JIA, oJIA). This classification might however reflect disease activity instead of depicting homogenous groups of patients. Large cohort studies have tried to find and characterize homogeneous groups of JIA patients independent of current classification and found that early-onset, ANA-positive arthritis is a well-defined entity. [87-90] Cytokine profiles are a (partial) reflection of the inflammatory process and could aid in distinguishing biologically different homogeneous JIA categories. Indeed, the homogenous group of early-onset (<6 years of age at disease onset) ANA-positive JIA patients showed a distinct cytokine profile supporting the suggested reclassification of JIA types. Besides clinical studies, genetic studies showed that the first 6 years of life are an important determinant in the type of disease. [91] For instance, major histocompatibility complex genes contribute to the risk of disease and these have a window of effect that is limited to the first 6 years of life. [91] Furthermore, peripheral blood mononuclear cell gene expression analysis reveals biologic differences based on the age of disease onset instead of the number of joints involved. In this younger group (<6 years) a high proportion of ANA-positive patients was noted. [92] It for now unknown what triggered the autoreactivity towards antinuclear antigens and if this trigger is homogenous as they show an overlapping clinical and cytokine profile. As these children have an early-onset of disease a genetic susceptibility is plausible that seemed to be skewed towards autoreactivity of antinuclear antigens. In these young JIA patients, no reports of extensive tissue damage or inefficacy of self-debris clearance is described, as seen in SLE.[93] Could it therefore be that instead of increased exposure the threshold for self-antigen activation is lowered? Genome-wide association studies in RA and JIA have repeatedly reported an association with PTPN2 (protein tyrosine phosphatase, non-receptor type 2) and JIA and RA (among others). [94-97] Recently, it has been shown that PTPN2 is elevated in naive T cells leaving the thymus to restrict homeostatic T-cell proliferation and

prevent excess responses to self-antigens in the periphery. [98] While PTPN2-deficient T cells undergo rapid lymphopenia-induced homeostatic proliferation (LIP) when transferred into lymphopenic hosts and result in the development of autoimmune disease. [98] It could therefore indeed be that the threshold for self-antigen recognition and subsequent activation is lowered in JIA/RA patients with PTPN2 SNPs (Single Nucleotide Polymorphisms) resulting in autoimmune disease in the setting of normal auto-antigen exposure. Besides direct recognition of self-antigens resulting in autoimmune disease, infection has been postulated to be a trigger for JIA. [99-101] In the light of lowered TCR-threshold triggering, this might hypothetically also result in an altered cross-reactivity response in patients with PTPN2 SNPs. As during infection certain TCRs might now cross-react with self-antigens showing overlap with pathogens, resulting in increased autoreactivity. It is unknown if these early-onset ANA-positive children are indeed enriched for alterations in the PTPN2 locus and function.

The majority of biomarkers are focused on disease activity and severity, as described in chapter 9. Biomarkers for treatment response and susceptibility that help clinicians make initial treatment decisions are lacking or insufficient, yet required for optimal control of JIA. The use of biomarkers and clinical information seems to be necessary to classify a complex immune disease, such as JIA. For example, the subgroup of JIA-patients that are young age at disease onset and are ANA-positive have a higher risk of developing iridocyclitis and warrants a different clinical and therapeutic approach. Hereby, getting a step closer to patient-tailored medicine.

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ADDENDUM

NEDERLANDSE SAMENVATTING

Het afweersysteem

Het afweersysteem bestaat uit een aangeboren en verworven, adaptieve, component dat weerstand biedt tegen ziekteverwekkers, zoals o.a. bacteriën en virussen. Het aangeboren afweersysteem berust op herkenning van evolutionair geconserveerde componenten die kenmerkend zijn voor ziekteverwekkers. De cellen en componenten van het aangeboren afweersysteem reageren zeer snel op gevaar. Het adaptieve afweersysteem is veel trager, maar ook veel specifieker. Het adaptief immuunsysteem is een lerend systeem dat heel specifiek bepaalde componenten (antigenen) van de ziekteverwekker herkend en hierna resulteert in een immunologisch geheugen. Dit principe van een immunologisch geheugen is bijvoorbeeld essentieel bij vaccinaties en hierop volgende bescherming. Het adaptief immuunsysteem kan grofweg worden ingedeeld in twee compartimenten; B cellen, die met hulp van T cellen verantwoordelijk zijn voor de humorale reactie middels antilichamen (antistoffen), en T cellen die verantwoordelijk zijn voor celgemedieerde afweer.

De ontwikkeling van het adaptieve afweersysteem

De ontwikkeling van B- en T-cellen (lymfocyten) vindt respectievelijk plaats in het beenmerg en de thymus (zwezerik) en is onderhevig aan strenge selectie criteria. Deze strenge selectie, wat maar een fractie van de lymfocyten overleeft, leidt tot B- en T-cellen die in staat zijn om lichaamsvreemde componenten te herkennen en je te beschermen tegen ziekteverwekkers. Belangrijk is hierbij om geen lichaamseigen eiwitten te herkennen, omdat dit kan leiden tot auto-immuun ziektes zoals bijvoorbeeld reumatoïde artritis. Zolang lymfocyten hun te herkennen antigeen nog niet zijn tegen gekomen worden zij beschouwd als naïeve cellen. Zodra ze wel hun antigeen hebben gevonden zullen ze zich vermeerderen en differentiëren tot effector-cel noodzakelijk voor de afweerreactie. Hierna zal het overgrote deel van de geactiveerde cellen sterven en een deel zal zich ontwikkelen als geheugen-cel die belangrijk is voor het immunologisch geheugen, zodat een volgende ontmoeting met hetzelfde antigeen sneller en beter verloopt.

De regulatie van het afweersysteem

Om te zorgen dat een afweerreactie na activatie weer tot rust komt en niet buitensporig verloopt en hiermee tot onnodige schade aan jezelf leidt, wordt deze nauwkeurig gecontroleerd door verschillende factoren. Een belangrijke rol voor onderdrukking van afweerreacties is weggelegd voor de regulatoire T cel (Treg). Deze cellen spelen een belangrijke rol in de balans van afweerreacties. Een verlies van deze rol kan bijvoorbeeld leiden tot overmatige afweer reacties tegen ziekteverwekkers, maar ook tegen het eigen lichaam. Een overmaat van de onderdrukking door Treg cellen wordt onder andere gezien in kanker, waarbij deze Treg cellen de afweerreactie van het eigen lichaam tegen de kanker

onderdrukt. Een delicate balans tussen zelf en niet-zelf is dus vereist van de functie van Treg.
Niet alleen de sterkte van de onderdrukking door de Treg, maar ook de stabiliteit van de Treg cel is van belang. De mate van stabiliteit wordt gemeten door te kijken naar het aantal methyl-eiwitten die gebonden zijn aan FOXP3. FOXP3 is een essentiële factor voor Treg cellen die verantwoordelijk is voor de functie. Als er minder methyl-groepen (demethylering) aan FOXP3 zitten dan is deze Treg cel stabieler. Minder stabiele Treg cellen kunnen minder goed afweerreacties onderdrukken en worden onder andere vaker gezien bij auto-immuunziekten. Deze extra informatie over Treg stabiliteit heeft meer inzicht geleverd betreft de functie van Treg cellen in gezondheid en ziekte. Echter, is voor deze meting een grote hoeveelheid T-cellen en dus bloed nodig waardoor het minder geschikt is voor kinderen waarbij slechts kleine hoeveelheden bloed afgenomen kan worden. In *hoofdstuk 4* beschrijven we een nieuwe techniek die het nu ook mogelijk maakt om bij kleine hoeveelheden bloed Treg stabiliteit te meten. Hiermee kan er verder inzicht worden verkregen in de ontwikkeling en stabiliteit van de Treg cel in ziekte en gezondheid bij kinderen.

DE ROL VAN DE THYMUS IN T CEL HOMEOSTASE EN HEROPBOUW

De rol van de thymus in T cel homeostase
De grootte van lymfocyten populaties blijft relatief stabiel tijdens veroudering en is bij T cellen door toedoen van twee belangrijke mechanismen die compenseren voor cel verlies: (1) de generatie van nieuwe T cellen uit de thymus (zwezerik), en (2) de expansie van al aanwezig T cellen (homeostatische proliferatie). De thymus is uitermate belangrijk voor het maken van nieuwe T-lymfocyten. Al rond de 16^{e} zwangerschapsweek begint de thymus nieuwe T-cellen te produceren. Rond het eerste levensjaar begint de productie af te nemen totdat nog slechts een kleine restfunctie aanwezig is na de puberteit en het T-cel aantal voornamelijk nog onderhouden door homeostatische proliferatie. De groep van T-cellen die uit de thymus komen hebben een grote verscheidenheid aan T-cel receptoren (TCR) die een verschillend antigeen herkennen en hierop reageren. In geval van homeostatische proliferatie is er echter een vermenigvuldiging van een bestaande T-cel populatie waardoor er geen nieuwe TCR reactiviteit ontstaat. Specifieke T-cellen kunnen zelfs uitgroeien indien deze preferentieel gestimuleerd worden. Bij jonge kinderen wordt vaak een grote diversiteit gezien van T-cel reactiviteit terwijl bij het ouder worden de diversiteit afneemt en een bepaalde reactiviteit ondervertegenwoordigd kan worden. Deze 'gaten in het immuunsysteem' kunnen een mogelijke verklaring zijn voor de verhoogde infectiekans bij ouderen.

T cel heropbouw in afwezigheid van de thymus is niet zonder consequenties

Indien er weinig T cellen aanwezig zijn in het immuunsysteem (lymfopenie) zal er ter compensatie homeostatische T cel proliferatie en verhoogde thymus productie optreden. Homeostatische proliferatie is echter vele malen sneller in het 'vullen' van het immuunsysteem dan de thymus. Zoals eerder aangegeven, resulteert dit wel in een lagere diversiteit van T cellen door de expansie op basis van specifieke antigenen. Er zijn aanwijzingen dat de preferentieel geëxpandeerde T cellen ook mogelijk gestimuleerd zijn door antigenen van het lichaam zelf. Dit kan mogelijk auto-immuunziekten tot gevolg hebben door de expansie van zelf-reactieve T-cellen. Een kenmerk van auto-immuunziekten is dat er vaak auto-antilichamen detecteerbaar zijn door de verhoogde reactiviteit tegen specifieke zelf-antigenen. In *hoofdstuk 5* kijken we naar de ontwikkeling van het auto-antilichaam profiel van kinderen waarbij op jonge leeftijd de thymus is verwijderd en het immuunsysteem dus alleen afhankelijk is van homeostatische proliferatie ter compensatie van T-cel verlies. In 2/3 van de kinderen waarbij de thymus is verwijderd werden op latere leeftijd auto-antilichamen gemeten. Dit is aanzienlijk hoger dan bij gezonde kinderen (~20%). Deze auto-antilichaam ontwikkeling blijkt voornamelijk samen te hangen met T cel homeostatische proliferatie op de jonge leeftijd. Ondanks dat de meeste kinderen en jong volwassenen auto-antilichamen hebben ontwikkeld zijn er geen klinische klachten van auto-immuunziekten. In *hoofdstuk 2 en 5* beschrijven we dat er ook preferentiële expansie is van Treg cellen na thymectomie, die deze auto-immuun reacties potentieel kunnen onderdrukken voordat ze klachten geven. Het is echter vooralsnog onduidelijk of deze kinderen een verhoogd risico lopen op het ontwikkelen of vroegtijdig ontwikkelen van auto-immuunziekten. Deze kinderen verdienen daarom extra aandacht bij klachten passend bij auto-immuunziekten.

De thymus is verantwoordelijk voor naïeve T cel productie en functie

De thymus is verantwoordelijk voor de productie van nieuwe (naïeve) T cellen. Deze naïeve T cellen zijn in te delen in twee groepen op basis van de expressie van CD31. CD31 positieve (+) naïeve T cellen worden gezien als cellen die recent de thymus hebben verlaten, terwijl de CD31 negatieve (-) naïeve T cellen al een aantal celdelingen hebben gemaakt zonder dat ze tot effector T cel zijn gedifferentieerd. In *hoofdstuk 3* bekijken we beide naïeve T cellen bij kinderen waarbij de thymus in de eerste levensmaand is verwijderd. Bij deze kinderen zien we dat het aantal CD31+ naïeve T cellen afnemen en de CD31- naïeve T cellen relatief toenemen. Dit kan te verklaren zijn door de afwezigheid van nieuwe naïeve T cel aanmaak na thymus verwijdering maar ook doordat CD31+ naïeve T cellen mogelijk zijn overgegaan tot de CD31- populatie. Bij nader onderzoek van CD31+ naïeve T cellen bij gethymectomeerde kinderen blijkt echter dat deze cellen ondanks hun naïeve fenotype zich niet gedragen als CD31+ naïeve T cellen. Ze produceren zeer weinig van een specifiek signaleringseiwit (interleukine 8, IL-8) en reageren minder sterk op activatie zoals bij

CD31+ naïeve T cellen van gezonde kinderen. Bij aanvullend onderzoek bleek dat de CD31+ naïeve T cel is onder te verdelen in twee functioneel verschillende groepen op basis van IL-8 productie. De IL-8 producerende naïeve T cel bleek hierbij erg afhankelijk van de thymus, want bij teruggroei van de thymus trad ook een functioneel herstel op van de IL-8 productie in CD31+ naïeve T cellen. Dit hoofdstuk stelt hiermee een nieuwe onderverdeling voor van naïeve T cellen dat verder inzicht biedt voor de ontwikkeling van het T cel immuunsysteem.

De rol van de thymus in de heropbouw van het immuunsysteem bij stamcel transplantatie

Stamceltransplantatie is een behandeling dat onder andere wordt verricht voor ernstige auto-immuunziekten. Met deze behandeling kunnen kinderen in tegen stelling tot medicatie volledig van hun ziekte genezen of langdurig zonder ziekte zijn. Stamceltransplantatie wordt vooraf gegaan met verwijdering van het immuunsysteem (door chemotherapie, bestraling) waarna in de autologe setting de stamcellen van de patiënt opnieuw worden ingebracht die dan een nieuw immuunsysteem moeten opbouwen. In de allogene setting worden stamcellen van een ander persoon gegeven die immunologisch erg lijkt op die van de patiënt.

Er wordt gedacht dat het nieuwe T cel systeem wordt opgebouwd door de thymus. Echter blijven er altijd wel enige T cellen achter na chemotherapie of bestraling, die mogelijk nog geassocieerd zijn met de auto-immuunziekte. Het herleiden van de bijdrage van de thymus of van homeostatische proliferatie van de overgebleven T cellen bij de heropbouw van het immuunsysteem is in de autologe setting bij mensen niet te maken, omdat de T cellen van dezelfde persoon afkomstig zijn en dus op elkaar lijken. In *hoofdstuk 7*, gebruiken we een muizenmodel waarbij we met specifieke markers op cellen konden herleiden wat de origine (stamcel of achtergebleven) van de cel was. De muizen hadden artritis en kregen hiervoor een beenmerg transplantatie. De heropbouw van de T cellen bleek initieel gemedieerd door de overgebleven T cellen, maar werd daarna snel overschaduwd door de T cellen uit de thymus. De nieuwe T cellen uit de thymus waren ook niet met meer reactief tegen eiwitten betrokken bij de artritis in muizen zoals gezien werd voor de transplantatie, wijzend op een nieuw opgeleid immuunsysteem en niet expansie van de overgebleven T cellen. De muizen hadden ook veel minder artritis na de transplantatie. Dit bleek ook voor de Treg cellen het geval, want deze waren meer stabiel en beter in het onderdrukken van afweerreacties dan de Treg van achtergebleven cellen (*hoofdstuk 6*). Het behandelen van de muizen met extra Treg cellen zodat ze helemaal geen ontstoken gewrichten meer hadden na de transplantatie had geen toegevoegde waarde en resulteerde zelfs in het onderdrukken van nieuwe T cel aanmaak. In enkele kinderen die een autologe stamceltransplantatie kregen voor een auto-immuunziekte hebben we geprobeerd onze resultaten bij de muizen te vertalen naar de mens. Bij nieuwe T cellen van de thymus ontstaat er een potentiele nieuwe

T cel reactiviteit in plaats van expansie van een al bestaande reactiviteit zoals bij homeostatische proliferatie. Na stamceltransplantatie bij kinderen zagen we meer nieuwe reactiviteit ontstaan van T cellen en voornamelijk Treg cellen. Indien een kind niet goed reageerde op de stamceltransplantatie bleek ook dat de reactiviteit van de Treg cellen niet was toegenomen. Nieuwe T cel productie door de thymus en hierbij specifiek de generatie van nieuwe Treg cellen lijkt uitermate belangrijk voor een gezonde heropbouw van het immuunsysteem na stamcel transplantatie.

DEEL TWEE

Her-classificering van de auto-immuunziekte jeugdreuma is nodig

Afhankelijk van het doel van de auto-reactiviteit van het immuunsysteem zijn er vele verschillende auto-immuunziekten mogelijk. De oorzaak van deze auto-reactiviteit en de ontwikkeling tot auto-immuniteit is bij vele gevallen onduidelijk. Dit is ook het geval bij juveniele idiopathische artritis (JIA, jeugdreuma) en volwassen reumatoïde artritis (RA). Er zijn vele biologische kenmerken (biomarkers) onderzocht en bekeken bij RA die geassocieerd zijn met ziekte-activiteit, ernst, behandelingsgevoeligheid en prognose die proberen homogeniteit te creëren binnen een heterogene ziekte. Vele verschillende biomarkers zijn onderzocht en worden samengevat in *hoofdstuk 8*. In het geval van JIA worden de kinderen in een bepaalde categorie geclassificeerd op andere de basis van het aantal ontstoken gewrichten. Indien er minder dan 5 gewrichten zijn betrokken heeft het kind de oligoarticulaire vorm, terwijl bij 5 of meer gewrichten het kind polyarticlair JIA heeft. Er wordt gesuggereerd dat deze classificatie echter meer de mate van ziekteactiviteit weergeeft en niet van een verschillend subtype JIA. Op basis van verschillende klinische kenmerken en een antinucleair antilichaam (ANA) blijken kinderen met JIA beter te classificeren. Echter ontbrak er nog een immunologische ondersteuning van deze nieuwe classificatie. In *hoofdstuk 9* hebben we een groot panel van biomarkers gemeten in het plasma van kinderen met JIA. Hieruit bleek dat de nieuwe classificatie van JIA ook een overeenkomstig biomarker profiel had, terwijl dit niet het geval was bij de oude classificatie. Deze jonge kinderen die ANA+ zijn blijken dus een relatief homogene groep van kinderen met JIA. Door de voorgestelde nieuwe indeling kunnen kinderen met JIA mogelijk beter benaderd worden, want kinderen met een vroeg begin van JIA (leeftijd <6 jaar) met ANA+ auto-antilichamen blijken een grotere kans te hebben op oogontsteking bij hun jeugdreuma.

Tot slot worden al deze bevindingen samengevat en bediscussieerd in hoofdstuk 10, waarbij nog kort de noodzaak van onderzoek in kinderen en de samenhang tussen de microbiologie en het afweersysteem worden besproken.

Conclusie

In dit proefschrift beschrijven we de dynamiek van de T cel heropbouw van het immuunsysteem bij kinderen met zijn mogelijke consequenties. Snel herstel van een laag aantal T cellen is uitermate belangrijk om adequaat te reageren op ziekteverwekkers. De thymus speelt een belangrijke rol in de heropbouw van een gezond immuunsysteem maar verloopt traag. Homeostatische proliferatie is relatief snel in T cel herstel, maar heeft het potentieel om het gestoorde immuunsysteem te behouden. Dit blijkt o.a. uit het ontstaan van auto-antilichamen na thymectomie en ziekte terugval bij lage Treg reactiviteit na stamceltransplantatie ondanks adequate T cel aantallen. Uiteindelijk lijkt het immuunsysteem je de eerdere lage T cel aantallen dus wel te vergeven, maar niet vergeten.

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CURRUCILUM VITAE

Theodorus van den Broek was born in Amersfoort on January the 3rd in 1980. After his graduation he moved to Utrecht to study medicine at the University of Utrecht in 2000. During his medical study he worked part-time at the laboratory of experimental cardiology in Utrecht (supervised by Prof.Dr. Gerard Pasterkamp) on the Athero-express project. This sparked his interest in experimental research what resulted in a half-year research internship at the department of pediatric immunology (supervised by Dr. Alvin Schadenberg, Prof. Dr. Berent Prakken and Dr. Koos Jansen) in combination with his final clinical rotation at the Wilhelmina children's hospital (WKZ) in Utrecht at the pediatric immunology ward.
After graduating medical school in 2006 he started as a junior medical doctor (ANIOS) at the department of pediatrics for one year (supervised by prof. Jan Kimpen). He was then offered a visiting scientist position abroad under the supervision of Prof. Dr. Salvo Albani. During this two-and-half year period he mainly focused on epitope-specific immune therapy in rheumatoid arthritis at the University of California, San Diego (UCSD) (Oct 2007- Febr. 2008), Arizona Arthritis Center at the University of Arizona (UofA), Tucson (Febr. 2008-Jan. 2010) and finally at the Sanford-Burnham Medical Research Institute, San Diego (Jan. 2010-June 2010), sponsored by the Fulbright Association, the Prince Bernhard Culture Fund (via Carolus Magnus foundation), and a fellowship of the Arizona Arthritis Center.
On his return to the Netherlands he started his PhD project at the "Eijkman Graduate School for Infection and Immunity" at the UMC Utrecht, the Netherlands. He worked at the pediatric immunology department, now part of "Laboratory of Translational Immunology" (LTI), under the supervision of Dr. Femke van Wijk, Prof. Dr. Berent Prakken and Dr. Koos Jansen. During this PhD program, he focused on T-cell reconstitution after lymphopenia, specifically after stem cell transplantation and neonatal thymectomy. During his PhD he arranged a 2-month work visit to the Weizmann Institute of Science in Israel (under supervision of Prof. Dr. Irun Cohen), sponsored by the Royal Academy of Sciences (KNAW) and the Dutch Society of Immunology (NVvI), to assess the development of autoreactivity in the lymphopenic setting after neonatal thymectomy. The results of his research focusing on T cell reconstitution are presented in this thesis.
Currently, Theo started his training program/residency for medical microbiology at the University medical center Utrecht (UMCU) under the supervision of Dr. Annemarie Wensing and Prof. Dr. Marc Bonten.

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