A single nucleotide polymorphism in the *ENTPD1* gene determines CD39 expression levels in regulatory T cells and impinges on their function

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ABSTRACT

Regulatory T (Treg) cells use different pathways to suppress proliferation and cytokine production by other immune cells. One mechanism is the conversion of pro-inflammatory adenosine triphosphate (ATP) to immunosuppressive adenosine by the ectonucleotidases CD39 and CD73. In mice, all Treg cells express these two molecules on the cell surface, and CD39 deficiency results in impaired regulatory function and autoimmune disease. Interestingly, the frequency of human $CD39^+$ Treg cells is highly diverse even among healthy donors. The aim of my thesis was to assess how CD39 expression is regulated in Treg cells, and the functional consequences that differences in CD39 expression in Treg cells may have on disease.

In peripheral blood from adults, 2-70% of Treg cells express CD39. Activation of $CD39^-$ Treg cells results in modest upregulation of CD39, thus it cannot explain the high levels found in some donors. In addition, $CD39^+$ Treg cells already exist in the thymus and cord blood, and the expression level of CD39 on Treg cells in adult individuals is stable over many months, altogether indicating inherent regulation. Indeed, a genetic analysis revealed that expression levels of CD39 on Treg cells is determined by a single nucleotide polymorphism in its gene, *ENTPD1*. Functionally, $CD39^+$ Treg cells have a higher capacity to suppress responder T (Tresp) cell proliferation and especially to suppress the production of interleukin (IL)-17A and interferon-γ (IFN-γ), compared to their $CD39^-$ counterparts. Accordingly, Treg cells from donors with the AA genotype (which have low frequency of $CD39^+$ Treg cells) have a reduced capacity to suppress inflammatory cytokine production by Tresp cells compared to Treg cells from GG donors (high frequency of $CD39^+$ Treg cells). To evaluate the role of $CD39^+$ Treg cells in disease, I analyzed blood samples from patients with monoclonal B cell lymphocytosis (MBL) and chronic lymphocytic leukemia (CLL) and showed that disease progression is associated with an increased frequency of $CD39^+$ Treg cells. Further, the suppressive capacity of total Treg cells from patients with MBL and CLL is superior in advanced stages of the disease.

Taken together, this work demonstrates that the expression of CD39 on Treg cells is primarily genetically driven, and that the inter-individual differences have a functional consequence in the regulation of the immune response.

ZUSAMMENFASSUNG

Regulatorische T (Treg) Zellen können die Proliferation und Zytokinproduktion anderer Immunzellen auf verschiedene Art und Weise unterdrücken. Ein Mechanismus ist die Umwandlung von proinflammatorischem Adenosintriphosphat (ATP) in immunsuppressives Adenosin. Diese Umwandlung wird durch zwei ecto-Nukleotidasen katalysiert: CD39 baut ATP ADP und AMP ab und CD73 generiert Adenosin aus AMP. Die Bedeutung dieses Umwandlungsprozesses wurde anhand verschiedener Tiermodelle gezeigt. So weisen CD39-defiziente Treg Zellen eine Beeinträchtigung ihrer regulatorischen Funktion auf, was mit einem schwereren Krankheitsverlauf einhergeht. Interessanter Weise schwankt der relative Anteil an $CD39^+$ Treg Zellen stark von Mensch zu Mensch. Ziel dieser Doktorarbeit war es herauszufinden, wie die CD39 Expression in humanen Treg Zellen reguliert ist und wie sich die interindividuellen Unterschiede in der $CD39^+$ Treg Zell Frequenz auf den Verlauf von Erkrankungen auswirken.

Im Blut erwachsener Menschen exprimieren 2-70% der Treg Zellen CD39 und $CD39^+$ Treg Zellen konnten auch im Thymus junger Kinder nachgewiesen werden. Nach Aktivierung wird CD39 auf zuvor $CD39^-$ Treg Zellen nur schwach hochreguliert. Langezeituntersuchungen ergaben, dass die Frequenz der $CD39^+$ Treg Zellen von erwachsenen Probanden über einen Zeitraum von zwei Jahren nahezu gleich bleibt. All dies weißt auf eine intrinsische Regulation der CD39 Expression in Treg Zellen hin. Daraufhin durchgeführte genetische Analysen ergaben, dass die Expression von CD39 in Treg Zellen durch einen Einzel-Nunkleotid-Polymorphismus (SNP) im CD39-kodierenden *ENTPD1* Gen bestimmt wird. Funktionell unterschieden sich $CD39^+$ Treg Zellen von $CD39^-$ Treg Zellen durch eine effektivere Blockade der effektor T Zell Proliferation sowie durch die gezielte Unterdrückung der Produktion der proinflammatorischen Zytokine Interleukin-17A (IL-17A) und Interferon-γ (IFN-γ). Dementsprechend zeigen Treg Zellen aus Spender vom SNP Genotyp AA (geringer Anteil an $CD39^+$ Treg Zellen) eine verminderte Unterdrückung der Produktion von proinflammatorischem IL-17A und IFN-γ verglichen mit Treg Zellen aus Spendern vom SNP Genotyp GG (hoher Anteil an $CD39^+$ Treg Zellen). Um die Rolle der $CD39^+$ Treg Zellen im Verlauf von Krankheiten zu beurteilen habe ich Blutproben von Patienten mit monoklonaler B Zell Lymphozytose (MBL) und chronisch

lymphozytischer Leukämie (CLL) untersucht und konnte beobachten, dass mit fortschreitender Erkrankung der Anteil der $CD39^+$ Treg Zellen zunimmt. Zudem stieg die suppressive Kapazität der Treg Zellen aus MBL und CLL Patient mit dem Verlauf der Erkrankung.

Zusammengefasst zeigt diese Arbeit, dass die Expression von CD39 auf Treg Zellen primär genetische reguliert ist, und dass die interindividuellen Unterschiede mit einer funktionellen Modulierung des Immunsystems assoziiert sind.

TABLE OF CONTENT

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

During the course of millenniums mankind has developed strategic warfare and time has provided evidence that this is critical to victory. As Sun Tzun put it: "…it is said that one who knows the enemy and knows himself will not be endangered in a hundred engagements" (Sun and Sawyer, 1994). This statement can be transferred to the immune system, which preserves the integrity and function of primitive and higher species. The immune system displays a network of different cell populations and effector molecules and is classically divided into two units, innate and adaptive immunity.

1.1.1 INNATE AND ADAPTIVE IMMUNITY

The immune system faces the challenge to discriminate between "self" and "non-self". This is accomplished through the collaboration between innate immunity, available at birth, and adaptive immunity, acquired throughout life via the recognition of discrete antigens. Together, they efficiently protect against pathogens while tolerating the self-antigens of the host.

A variety of myeloid and lymphoid cells are part of the innate immune system. They are able to exert rapid effector functions through a limited repertoire of germline-encoded pattern-recognition receptors (PRRs) that recognize invariant pathogen-associated molecular patterns (PAMPs) (Schenten and Medzhitov, 2011; Vivier et al., 2011). Adaptive immunity, in contrast, depends on the generation of a diverse repertoire of antigen receptors expressed on T and B cells and subsequent activation and clonal expansion of cells carrying the appropriate antigen-specific receptors (Schenten and Medzhitov, 2011). These two mechanisms were thought to work completely independent, but as Charles A. Janeway hypothesized correctly, innate recognition of non-self is linked to the induction of adaptive immunity (Janeway, 1989). The generation of B and T cell responses depends on the induction of co-stimulatory molecules and the secretion of cytokines and chemokines initiated by

the cells of the innate immune system (Pasare and Medzhitov, 2005). After encounter with a pathogen, the innate immune system provides an immediate, but non-specific response. Additionally, this response activates the adaptive immune system, which efficiently takes over the protection against pathogens. The highly specific response of the adaptive immune cells is retained after elimination of the pathogen, creating immunological memory. Memory enables the adaptive immune system to enhance the response to subsequent encounters with the same pathogen. In this way, the adaptive immune system of higher vertebrates protects the organism from an ever-evolving array of pathogenic microorganisms, at the same time that immune tolerance to self is maintained (Fontenot et al., 2003).

1.1.2 THYMIC DEVELOPMENT OF CD4 AND CD8 T LYMPHOCYTES

The thymus is the primary lymphoid organ responsible for the differentiation of T cells. In newborn children the thymus is remarkably large and reaches its maximum size within the first 12 month of life. Over the course of time the volume of thymic tissue decreases progressively and is replaced by fat tissue (Good, 1968). During T cell development, thymocytes undergo a number of differentiation steps (Gill et al., 2003; Legrand et al., 2007).

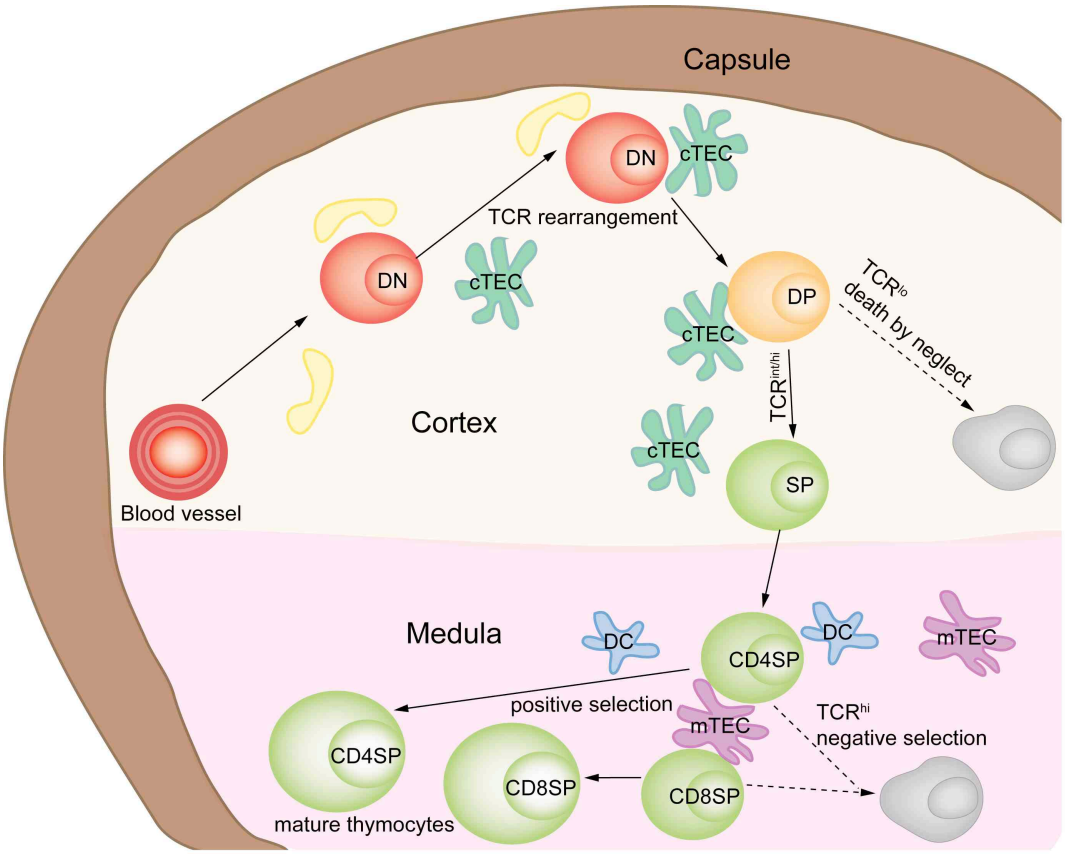


Figure 1.1 Thymic development of αβ T cells
The thymus is divided into two main anatomic regions, the medulla and the cortex. Bone marrow-derived precursor cells enter the thymus and migrate to the outer cortex. Interaction with stromal cells induces proliferation of double negative (DN, $CD4^{-}CD8^{-}$) thymocytes. These cells undergo T cell receptor (TCR) rearrangement and develop into double positive (DP, $CD4^{+}CD8^{+}$) thymocytes. Interaction with cortical thymic epithelial cells (cTEC) induces death by neglect of DP thymocytes with low affinity for self-peptide:MHCs. Surviving cells are positively selected and commit to either CD4 or CD8 lineage. These single positive (SP) cells migrate to the medulla where they scan medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) for self-peptides. Cells with high avidity for self are eliminated (negative selection). The surviving mature SP cells egress to periphery.

$CD34^{+}CD1A^{-}$ bone marrow-derived T cell precursors enter the thymus via the cortico-medullary junction, and migrate as $CD4^{-}CD8^{-}$ (DN) thymocytes outwards into the outer cortex region (Gill et al., 2003; Spits, 2002). Interaction of DN cells with thymic stromal cells initiates proliferation and differentiation of the DN cells (Figure 1.1). The acquisition of CD1a marks the commitment to the T cell lineage, and at this stage starts the rearrangement of the T cell receptor (TCR) genes. These cells will then acquire a functional TCRαβ dimer, and develop into $CD4^{+}CD8^{+}$ double positive (DP) cells. However, only the thymocytes whose TCR recognize self-peptide:MHC (Major

Histocompatibility Complex) on cortical thymic epithelial cells (cTECs) receive a survival signal (Daniels et al., 2006), ensuring that the T cell repertoire is self-MHC restricted. Thymocytes that do not express a functional TCR capable of binding to self-peptide:MHC die by neglect. At this stage, the developing thymocytes that are positively selected will commit to either CD4 or CD8 lineage (Singer et al., 2008). However, T cells recognizing self-peptide:MHC with high affinity are potentially dangerous, since they could trigger an immune response against self-antigens of the host. To avoid this, positively selected thymocytes migrate into the thymic medulla where they interact with self-peptide:MHC complexes presented by medullary thymic epithelial cells (mTECs) and dendritic cells (DCs). Cells with high affinity to self-peptide:MHC receive an apoptotic signal (negative selection), and are deleted from the T cell repertoire to ensure tolerance to self antigens (central tolerance). Surviving mature thymocytes will then leave the thymus and egress to periphery as naïve cells (Anderson and Jenkinson, 2001; Klein et al., 2011).

1.1.3 $CD4^+$ T CELL DIFFERENTIATION

$CD4^+$ T cells are basic players in the orchestration of the adaptive immune response: They support B cells to produce antibodies, enhance and maintain responses of $CD8^+$ T cells, regulate macrophage functions, and mediate immune responses against a wide variety of pathogenic microorganisms. To accomplish these very diverse purposes, $CD4^+$ T cells undergo a process of specialization driven by the nature of the task and by environmental cues. Differentiation of naïve $CD4^+$ T cells into effector cells is initiated upon contact of the naïve T cell with its cognate antigen presented by MHC class II molecules on the surface of an antigen presenting cell (APC). The cytokine milieu and the function of different specific transcription factors directs T cells to express distinct soluble mediators and surface molecules, which in turn support interactions with other immune cells (Murphy and Stockinger, 2010). Mosmann and Coffman showed that the formerly described T-helper 1 (Th1) (Tada et al., 1978) cells produced interferon (IFN)-γ, while the Th2 cells produced interleukin (IL)-4 but not IFN-γ (Mosmann et al., 1986). Now we know that depending on the cytokines produced by the APCs, a naïve

CD4$^+$ T cell develops into one of several T cell subsets, such as Th1, Th2, Th17, or peripherally derived Treg (pTreg) or type 1 regulatory (Tr1) cells (Figure 1.2).

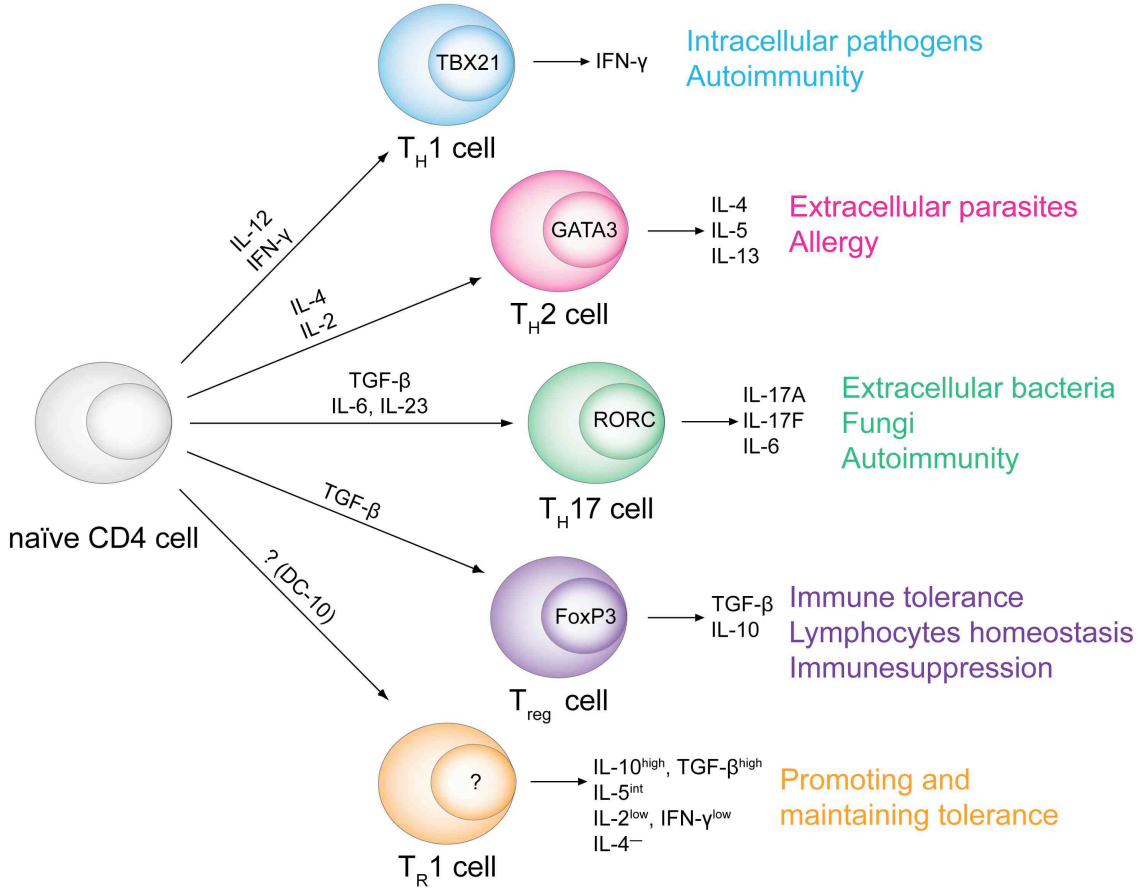


Figure 1.2 Differentiation of CD4$^+$ T cell effector subsets.
Upon activation, naïve CD4$^+$ T cells differentiate into Th1, Th2, T17, Treg or Tr1 cells. Each subset is characterized by the expression of a specific transcription factor, the production of certain cytokines, and holds a specific function within the adaptive immune system.

IL-12 triggers the induction of Th1 cells. These cells express TBX21 (murine homolog: T-bet) as lineage-specific transcription factor. Th1 cells promote a cell-mediated immune response to intracellular pathogens by the production of IFN-γ and activation of macrophages (Szabo et al., 2000). In contrast, Th2 cells are induced by IL-4, which stimulates the expression of the transcription factor GATA-3. Th2 cells organize humoral immunity against extracellular pathogens and secrete IL-4, IL-5 and IL-13 (Ouyang et al., 1998; Zheng and Flavell, 1997).

IL-23, a cytokine sharing a subunit with IL-12, is responsible for the maintenance of Th17 cells (Becher et al., 2002; Cua et al., 2003; Harrington et al., 2005; Oppmann et

al., 2000). Th17 cells express the lineage-specific transcription factor retinoic orphan receptor (RORC, murine homolog: RORγt) (Ivanov et al., 2006), and differentiate in the presence of tumor growth factor (TGF)-β, IL-6 and, in humans, IL-21 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). These cells have been termed Th17 cells due to their IL-17 production (Harrington et al., 2005; Park et al., 2005). As pro-inflammatory cytokine, IL-17 induces recruitment of neutrophils and triggers anti-microbial protein synthesis (Korn et al., 2009).

CD4 cells can differentiate not only into effector cells promoting pathogen clearance or inflammation, but also into cells that are able to modulate the function of other immune cell types. TGF-β triggers the differentiation of FoxP3-expressing peripherally-derived regulatory T (pTreg) cells from naïve $CD4^+$ T cells (Chen et al., 2003). The differentiation of both Th17 and Treg cells require TGF-β. In the presence of the pro-inflammatory cytokine IL-6, TGF-β induces the development of Th17 cells; in its absence pTreg cells producing IL-10 and IL-35 are favored. (Bettelli et al., 2006). Other regulatory $CD4^+$ T cell types can down modulate effector responses through soluble suppressive cytokines such as IL-10 and TGF-β, independently of cell-cell contact (Jonuleit and Schmitt, 2003), such as Th3 cells and Tr1 cells. Th3 cells produce TGF-β and express FoxP3 and, therefore, are likely to belong to the pTreg population (Carrier et al., 2007; Chen et al., 1994; Weiner, 2001). Tr1 cells, by contrast, do not express FoxP3 and secrete high amounts of IL-10 and TGF-β, intermediate amounts of IL-5, and low amounts of IL-2 and IFN-γ (Gol-Ara et al., 2012). Murine Tr1 cells are induced by IL-27. In humans, they arise through interaction with tolerogenic DC-10 (IL-10-producing DCs) (Gregori et al., 2010; 2012a). Although there is no unique marker for the characterization of human Tr1 cells, they express LAG3, CD49b and CD226, and are negative for CD25 and CD127 (Gagliani et al., 2013). Several transcription factors are known to regulate IL-10 expression, namely STAT3, C-MAF, and AhR. They all have been used to identify human IL-10-producing T cells, but it is still unclear if these transcription factors can be considered master regulators and *bona fide* markers for Tr1 cells (Gregori et al., 2012b). Therefore, whether Tr1 cells are a distinct $CD4^+$ T cell population or not is discussed controversially, since all $CD4^+$ T cells including Th1, Th2,

Th17 and Treg cells are capable of producing IL-10 (Zhu and Paul, 2008). The concept of terminally differentiated subsets of $CD4^+$ T cells as proposed by Mosmann and Coffman held for decades. However, there is accumulating evidence that these subsets show certain degree of plasticity. This means that differentiated $CD4^+$ T cell subsets have the ability to adapt to a new environment or a new pathogenic insult (Baxter and Jordan, 2013; Zhou et al., 2009a).

1.2 FOXP3$^+$ REGULATORY CD4$^+$ T CELLS

Gershon *et al.* discovered in the 1970s that T cells have the ability to downregulate immune responses of antigen-specific T cells (Gershon and Kondo, 1970). More than 20 years later, Sakaguchi *et al*. described high expression of CD25 as a marker for a subset of CD4 cells with regulatory function, which were named T-regulatory cells (Sakaguchi et al., 1995). These cells express the transcription factor forkhead box P3 (FoxP3), the master regulatory gene for cell-lineage commitment and developmental differentiation of Treg cells in the thymus, as well as in the periphery (Hori, 2003). Based on ontogeny, there are two $FoxP3^+$ Treg cell subsets: thymus-derived regulatory T (tTreg) cells and peripherally derived regulatory T (pTreg) cells (Figure 1.3), which are induced in the periphery in response to antigen stimulation under tolerogenic conditions. The key role of Treg cells in general is the control of immune responses and inflammatory reactions. In humans, Treg cells represent a small subset of $CD4^+$ T cells (around 5%) that interact with and suppress effector cells. Differences of tTreg and pTreg cells suppressor functions are not well characterized to date, most likely due to a lack of specific markers to differentiate between those two cell populations. However, these cells differ in their epigenetic modifications of the Treg-specific demethylated region (TSDR): tTreg cells have a completely demethylated TSDR, whereas the TSDR of pTreg cells is methylated (Baron et al., 2007; Floess et al., 2007; Kim and Leonard, 2007). Preliminary data has shown that tTreg cells possess a killing ability similar to NK cells to suppress B cell responses, while pTreg cells suppress B cells independently of this killing ability (Lin et al., 2013).

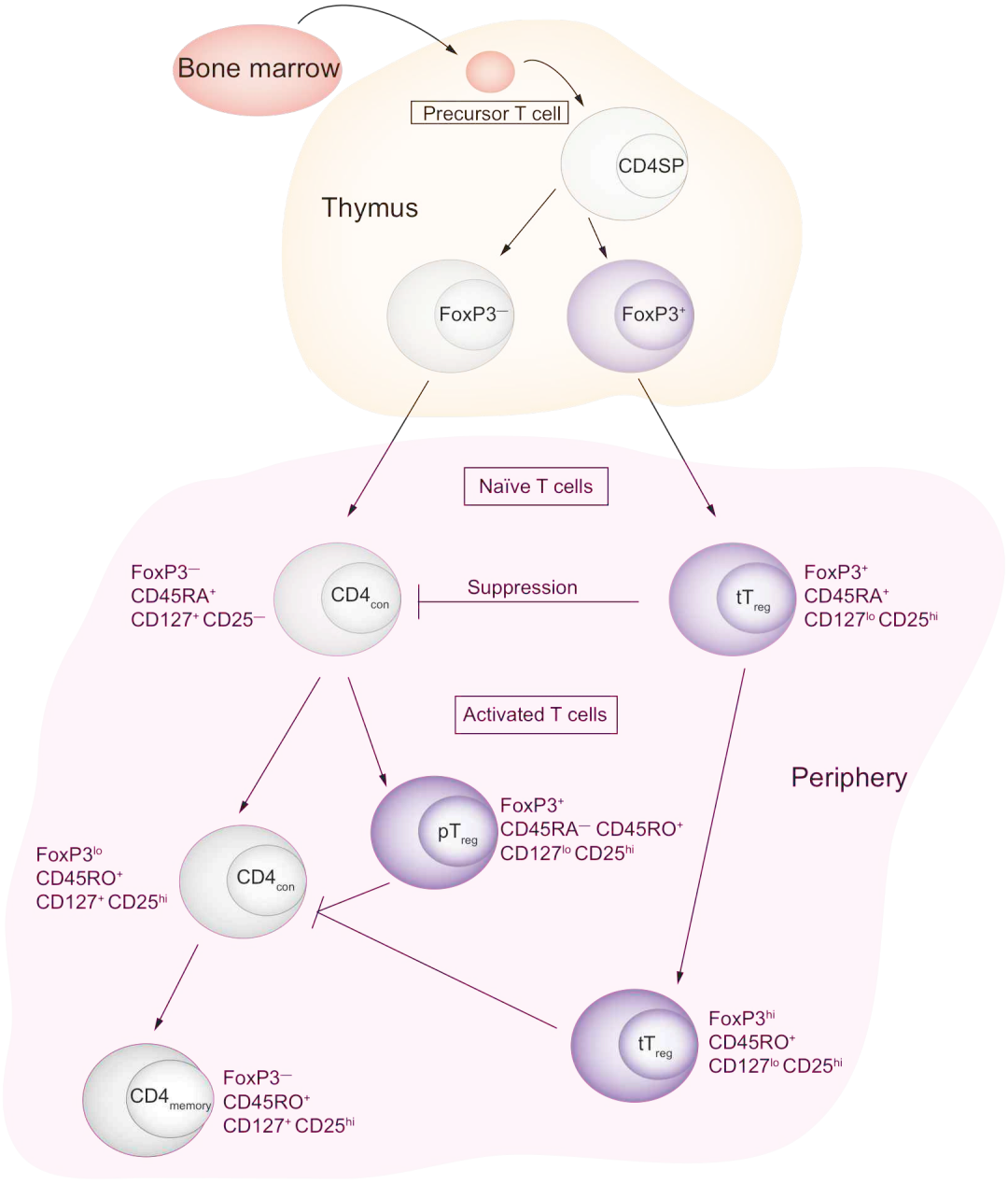


Figure 1.3 Ontogeny of CD4 conventional and regulatory T cells
CD4 sinlge positive (SP) cells develop in the thymus. These CD4 SP cells leave the thymus as $FoxP3^+$ thymus-derived Treg (tTreg) cells or $FoxP3^-$ CD4 conventional T (CD4con) cells. $FoxP3^-$ CD4con cells can convert into $FoxP3^+$ peripherally derived Treg (pTreg) cells.

1.2.1 THYMIC DEVELOPMENT OF TREG CELLS

The thymus is essential for the establishment and renewal of the peripheral T cell compartment with a diverse repertoire, able to efficiently respond to pathogenic insults, yet tolerating self-antigens. Central (thymic) tolerance is achieved by means of negative selection, however, it is not perfect, and some of the autoreactive cells

escape to periphery. Peripheral tolerance, by means of Treg cells, is responsible for suppressing autoreactive T cells that escaped negative selection. In the periphery they act together with pTreg cells to regulate effector responses (Figure 1.3).

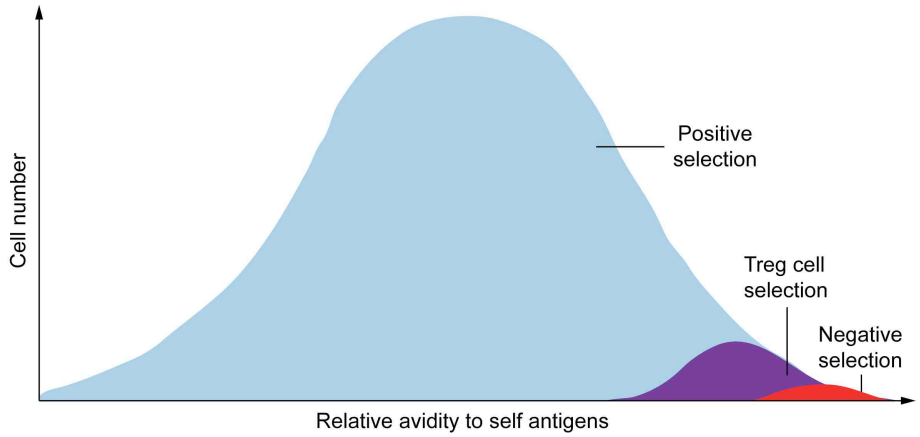


Figure 1.4 Thymic selection of tTreg cells.
During thymic differentiation, thymocytes undergo positive and negative selection. In the avidity model of thymic selection, thymocytes with strong avidity for self-antigens, are either negatively selected (red) or undergo Treg cell differentiation (purple). Adapted from *Hsieh et. al.* Nat Rev Immunol. 2012

The exact mechanisms by which tTreg cells develop are still controversial. Data from TCR-transgenic mice suggested that thymocytes with a borderline high affinity for self-antigens, but yet not negatively selected, develop into tTreg cells (Klein et al., 2009; Maloy and Powrie, 2001). This range of affinities confer the so-called 'agonist selection', and include not only the tTreg cells but also other cell types, such as nTh17 (Cheroutre et al., 2009). The dependence of tTreg cell selection on self-reactivity influences the T cell development in different ways. It limits the export of self-reactive T cells and it creates a regulatory T cell population, which can be activated by self-antigens in the periphery (Hsieh et al., 2012).

1.2.2 PHENOTYPE OF $CD4^+$ REGULATORY T CELLS

$CD4^+$ Treg cells and $CD4^+$-non Treg cells (referred to as conventional $CD4^+$ T cells, CD4con) can be discriminated through the expression of specific markers. Treg cells express high levels of the IL-2 receptor α-chain (CD25) (Dieckmann et al., 2001; Gershon and Kondo, 1970; Jonuleit et al., 2001; Levings et al., 2001). Together with the IL-2 receptor β-chain (CD122) and the common γ-chain (γ_c, CD132) these three subunits form the high affinity IL-2 receptor (IL-2R) (Hori, 2003; Minami et al., 1993),

which is expressed on Treg cells (Baecher-Allan et al., 2001; Malek and Bayer, 2004). Treg cells require IL-2 for their development (Jonuleit and Schmitt, 2003; Malek, 2008; Sakaguchi, 2004), but since they do not produce it, they depend on IL-2 produced by other cells (Gregori et al., 2012a; Malek and Bayer, 2004; Roncarolo et al., 2001). Resting CD4con cells do not express CD25, but upregulate this receptor upon activation and simultaneously produce IL-2 (Malek and Ashwell, 1985). Hence, it is not possible to discriminate Treg cells and activated CD4con cells on the basis of CD25 expression. However, human Treg cells consistently express lower levels of CD127 compared to the majority of other $CD4^+$ T cells (Seddiki et al., 2006). CD127 is the α-chain of the IL-7 receptor (IL-7Rα), and forms together with the γ_c the IL-7 receptor (IL-7R) (Kroemer and Richards, 1996). Binding of IL-2 to its receptor rapidly represses CD127 expression and at the same time it induces CD25 expression (Liao et al., 2013; Xue et al., 2002). Through the combined analysis of CD25 and CD127 it is possible to distinguish between Treg cells ($CD4^+CD127^{lo}CD25^{hi}$) and CD4con ($CD4^+CD127^+CD25^{lo}$) cells (Liu et al., 2006). Only the $CD4^+$ T cell subset expressing the highest levels of CD25 ($CD25^{hi}$) have suppressive activity *in vitro* (Baecher-Allan et al., 2001; Weiner, 2001).

Helios is another transcription factor expressed specifically in Treg cells, and in mice it is exclusively expressed by tTreg (Thornton et al., 2010). However, in human cells Helios can be upregulated in both $CD4^+$ and $CD8^+$ T cell subsets upon stimulation, independent of FoxP3 expression (Serre et al., 2011). Not all induced Treg cells co-express Helios and FoxP3, nevertheless, Helios is expressed in some $FoxP3^+$ pTreg cells.

Upon activation, $FoxP3^+$ Treg cells selectively express GARP (Glycoprotein A Repetitions Predominant), an orphan toll-like receptor that acts as an anchor for latent TGF-β by tethering the inactive cytokine to the cell surface. $FoxP3^+$ Treg cells express latent TGF-β complexed with the latency-associated peptide (LAP). The LAP-TGF-β complex binds to the GARP receptor on activated Treg cells (Stockis et al., 2009; Tran et al., 2009; Wang et al., 2009) and is shuttled to the cell surface, allowing the release of mature TGF-β (Battaglia and Roncarolo, 2009; Stockis et al., 2009).

The expression of FoxP3 is essential for Treg cell development and function (Fontenot et al., 2003). The importance of FoxP3 is illustrated by a rare genetic disease known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). In IPEX patients, mutations in the *FOXP3* gene result in a loss of regulatory cell function leading to a overt autoimmunity and eventually to early death (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Binding of Foxp3 to the promoter region of its target genes leads to increased (i.e. *CD25*, *CTLA-4*) or decreased histone acetylation (i.e. *IL-2*, *IFN-γ*) influencing transcription and thus supporting the developmental stability and the suppressive function of Treg cells (Chen et al., 2006; Marson et al., 2007; Sadlon et al., 2010). Furthermore, Treg cells have a specific DNA demethylation and histone modification motif in the conserved non-coding region 2 (CNS2) of the *FOXP3* gene (Ohkura et al., 2013). Demethylation of the *FOXP3* CNS2 region is important for inducing or stabilizing FoxP3 expression (Kim and Leonard, 2007; Polansky et al., 2010; Zheng et al., 2010). A complete demethylated CpG motif is exclusively observed in tTreg cells but not in activated CD4con cells or in TGF-β induced Treg cells (Baron et al., 2007; Floess et al., 2007; Kim and Leonard, 2007).

1.2.3 IMMUNOSUPPRESSIVE MECHANISMS OF $CD4^+$ REGULATORY T CELLS

Treg cells utilize different cell-contact dependent and independent mechanisms to suppress proliferation and/or cytokine production by other immune cells (some are depicted in Figure 1.5). One major cell contact independent suppressive mechanism is the secretion of immunosuppressive cytokines such as IL-10, TGF-β and IL-35. Further, Treg cells upregulate CD25 upon activation, which, as part of the high-affinity IL-2R, “consumes” IL-2 in the near periphery, thereby depriving the essential growth factor of CD4con cells (la Rosa et al., 2004). In addition, Treg cells participate in the conversion of pro-inflammatory adenosine triphosphate (ATP) into anti-inflammatory adenosine via the ectoenzymes CD39 and CD73. Contact dependent suppressive mechanisms of Treg cells comprise the ability to induce apoptosis of APCs or responder T (Tresp) cells through the secretion of granzyme and perforin (Wing and Sakaguchi, 2009). Moreover, Treg cells harbor high levels of cyclic adenosine monophosphate (cAMP), which they can transfer cAMP via gap junctions into effector T cells and suppresses

their activity. cAMP antagonists abolish this suppressive pathway (Bopp et al., 2007). Treg cells express the costimulatory molecule CTLA-4, which triggers the expression of the enzyme indolamine 2,3-dioxygenase (IDO) in dendritic cells (DCs) by interaction with CD80 and CD86 (Fallarino et al., 2003). IDO catabolizes the essential amino acid tryptophan to kynurenine and other metabolites, which are toxic to T cells neighboring the DCs (Munn et al., 2004). These signals can also promote nuclear localization of Foxo transcription factors, which suppress expression of genes encoding IL-6 and tumor necrosis factor P, phosphorylation (Wing and Sakaguchi, 2009).

1.2.4 REGULATORY T CELLS DEGRADE PROINFLAMMATORY ATP VIA CD39

CD39 (ectonucleoside triphosphate diphosphohydrolase 1, ENTPD1) is a dominant ectoenzyme in the immune system (Mizumoto et al., 2002) and is expressed on B cells, DCs and a subset of T cells. Initially the ectonucleotidase CD39 was described as an activation marker of lymphoid cells (Maliszewski et al., 1994). Kaczmarek *et al.* proposed that CD39 has anti-inflammatory action by hydrolyzing ATP and ADP, respectively, to AMP (Figure 1.5 B) (Kaczmarek et al., 1996). Five apyrase conserved regions in the molecule are essential for preservation of the hydrolytic activity (Drosopoulos et al., 2000). CD39 acts in concert with CD73 (ecto-5'-nucleotidase, NT5E), another ectonucleotidase present on the surface of lymphocytes to degrade AMP to adenosine (Airas et al., 1995; Dwyer et al., 2007). Treg cells use these ectoenzymes to generate immunosuppressive second messengers from nucleotides. (Figure 1.5 C).

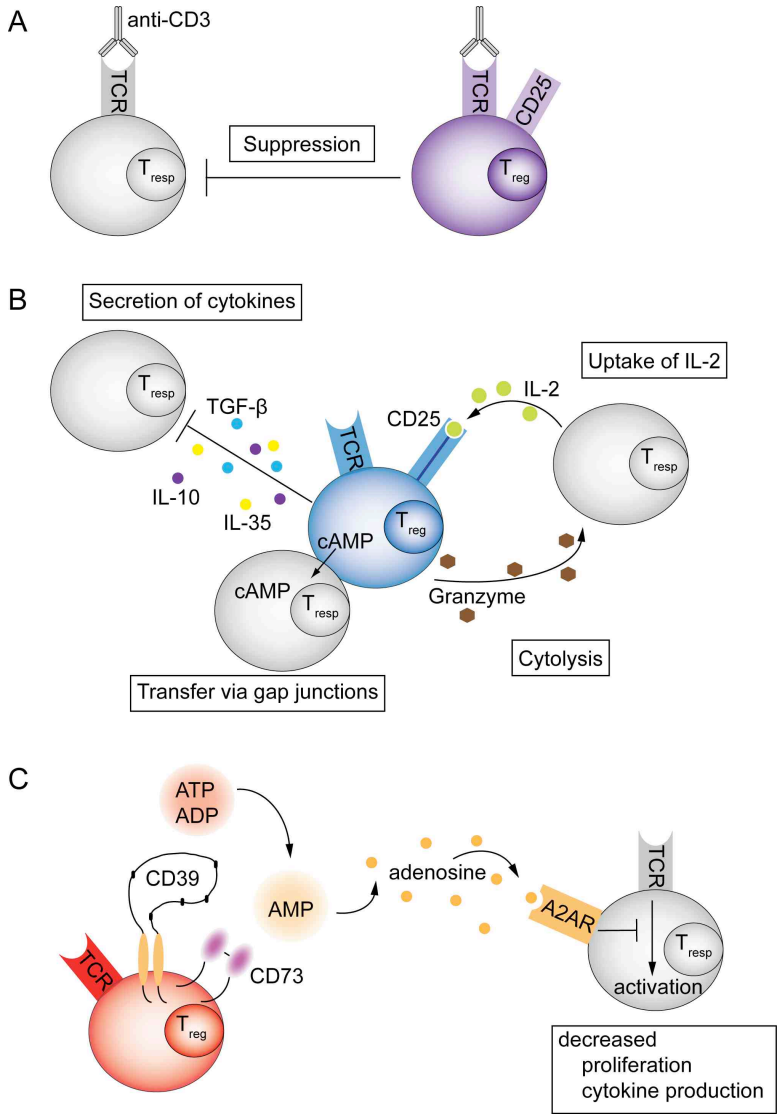


Figure 1.5 Schematic representation of Treg cell suppressive mechanisms.
(A) Schematic illustration of Treg cells suppressing Tresp cells. (B) Depicts suppressive mechanisms used by Treg cells. (C) Represents the mechanism by Treg cells expressing CD39 and CD73 to suppress proliferation of Tresp cells. Through the conversion of inflammatory ATP to AMP and the further degradation by CD73 to anti-inflammatory adenosine, which binds to adenosine 2A receptors on Tresp cells, suppression is induced.

In mice, CD39 and CD73 are co-expressed on virtually all Treg cells (Deaglio et al., 2007). In humans, by contrast, CD39 is expressed by a subset of Treg cells and CD73 is only found at low levels on Treg cells. Co-expression of both markers on Treg cell is rare (1-7%) (Mandapathil et al., 2010; Moncrieffe et al., 2010). These two ectoenzymes, CD39 and CD73, hydrolyze extracellular ATP to adenosine (Borsellino et al., 2007; Fletcher et al., 2009). The generated adenosine binds to adenosine receptors (i.e. adenosine A2 receptor, A2AR) on CD4con cells and elevates intracellular cAMP,

which diminishes their effector function (Zhang, 2010). Thereby, Treg cells are able to convert a pro-inflammatory signal (ATP) into an anti-inflammatory signal (adenosine). Adenosine can be degraded irreversibly to inosine by adenosine deaminase (ADA), which is ubiquitously expressed both intracellularly and on the cell surface. In humans it can form a complex on the cell surface with two molecules of CD26, whereas this complex does not form in mice (Dong et al., 1996; Kameoka et al., 1993; Schrader et al., 1990). $CD39^+$ Treg cells express low levels of CD26 and ADA. This is in direct contrast to CD4con cells which express low levels of CD39 but abundant levels of CD26 and ADA (Moncrieffe et al., 2010). Thus, the dynamics of the expression of these ectoenzymes and consequently the regulation of the levels of extracellular ATP, adenosine and intracellular cAMP in lymphocytes exert a fine-tuning effect on the modulation of the immune response and hence offer the opportunity for pharmacologic intervention. Likewise, $CD39^-$ cells show little or no ATPase activity, while all $CD39^+$ $CD4^+$ T cells, irrespective of FoxP3 expression, have rapid ATPase activity (Moncrieffe et al., 2010).

1.2.5 ROLE OF $CD39^+$ REGULATORY T CELLS IN DISEASE

Multiple sclerosis (MS) has been thought to be a Th1 cell driven autoimmune disease. In MS Th17 cells are less susceptible to suppression by Treg cells as Th1 cells, whereby during relapse the frequency of pathogenic Th17 cells increases, while Th1 cells remain stable. (Brucklacher-Waldert et al., 2009; Peelen et al., 2011). Treg cells from MS patients show normal numbers, but an impaired function (Haas et al., 2005; Venken et al., 2006; Viglietta et al., 2004). A more detailed analysis showed that the frequency of Treg cells expressing CD39 was reduced in MS patients, and the consequence was an impaired control of Th17 cells. In juvenile idiopathic arthritis (JIA), the synovial fluid of infected joints contains increased amounts of $CD39^+$ Treg cells, attenuating inflammation while it progresses. Numbers of $CD39^+$ Treg cells within the peripheral lymphocyte compartment are comparable between JIA patients and healthy donors (Moncrieffe et al., 2010).

Infection with the human immunodeficiency virus (HIV) alters immune cells and disturbs the balance of pro- and ant-inflammatory cytokines (Leal et al., 2005). During HIV infection, CD39 is specifically increased on Treg cells, which inhibit effector function and disable the reconstitution of the T cell pool, thereby contributing to disease progression (Nikolova et al., 2011; Schulze Zur Wiesch et al., 2011).

In cancer, Treg cells inhibit the effector lymphocytes and create a favorable environment for the growth of tumor cells. Ectonucleotidase-mediated production of adenosine contributes to the generation of this environment, leading to the inhibition of $CD4^+$, $CD8^+$ T cell and NK cell effector responses and resulting in tumor progression (Bastid et al., 2012). Among others, patients with B cell chronic lymphocytic leukemia (CLL) exhibit increased Treg cell numbers (D'Arena et al., 2012; Weiss et al., 2010). CLL is the most common leukemia in the western world and is characterized by clonal expansion of mature B cells in peripheral blood, lymphoid tissues and bone marrow (Dighiero and Hamblin, 2008). During progression of CLL, apart from clonal B cell expansion an expansion of T cells has also been monitored (Bagnara et al., 2011; Burger et al., 2009; Hoerning et al., 2011; Oo et al., 2010). CLL is preceded by a premalignant clonal B cell expansion, termed monoclonal B cell lymphocytosis (MBL), which is diagnosed in patients with <5000/µl peripheral monoclonal CLL-phenotype cells in the absence of other signs of lymphoma (Ghia and Caligaris-Cappio, 2012; Rawstron et al., 2010; Shanafelt et al., 2010). The progression to CLL among individuals with clinical MBL is very low (1-2% per year) (Rawstron et al., 2008; Shanafelt et al., 2009), and the events that initiate progression from clinical MBL to CLL remain unknown (Fazi et al., 2011). To date, it is not possible to predict the transition from MBL to CLL. Therefore, it is not possible to divide patients into low-risk MBL, who do not need clinical follow-up, and high-risk MBL, who should be clinically monitored like CLL patients. In CLL, T cells in general show dysfunctions which impairs their effector response against malignant CLL cells (Ramsay et al., 2012). This T effector cell dysfunctions in CLL patients is likely to be mediated by the expansion of Treg cells (D'Arena et al., 2011; Thornton et al., 2010).

During the course of different diseases, $CD39^+$ Treg cells hold a two-sided function: they can act beneficial (autoimmune disorders) or detrimental (HIV, cancer). Therefore, a pressing question is how the expression of CD39 is regulated in health and disease.

1.3 GOALS OF THE PROJECT

Treg cells are known to control immune responses and inflammatory reactions. In mice, virtually all Treg cells express the ATP degrading ectonucleotidase CD39, which acts in concert with CD73 to produce immunosuppressive adenosine. However, in the human system only a fraction of Treg cells expresses CD39. The goal of this thesis is to assess the role of CD39 in the function of Treg cells. Specific aims are:

- To perform a detailed characterization of the frequency and phenotype of $CD39^+$ Treg cells
- To compare $CD39^+$ Treg cells to $CD39^-$ Treg cells based on their function
- To find out how CD39 expression of Treg cells is regulated
- To determine the ontogeny of $CD39^+$ Treg cells
- To evaluate the contribution of $CD39^+$ Treg cells to the pathology of malignant diseases i.e. chronic lymphocytic leukemia (CLL)

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CELL CULTURE

Reagents for cell culture

Material	Company
Brefeldin A Solution, 1000x	eBioscience
Cell Proliferation Dye eFluor® 670	eBioscience
Dimethylsulfoxid (DMSO)	AppliChem
L-Glutamine, 200 mM	Gibco
Lymphocyte Separation Medium	PAA
Pan T Cell Isolation Kit II	Miltenyi Biotec
Penicillin/Streptomycin, 100x	PAA
RPMI 1640	Gibco
Serum, fetal bovine	Biochrom AG
Serum, human type AB	PAA
Trypan blue solution, 0,4%	Sigma Aldrich
X-VIVO 15, serum free medium	Lonza

Composition of media

Buffer	Compounds
Freezing medium	40% RPMI 40% FCS (heat inactivated) 20% DMSO
Standard RPMI medium	10% FCS (heat inactivated) 1% Penicillin/Streptomycin 2 mM L-Glutamine in RPMI
Treg expansion medium	5% hSerum (heat inactivated) 1% Penicillin/Streptomycin 2 mM L-Glutamine in RPMI

Cell stimulation & blocking reagents

Material	Company
ARL67156	Tocris
anti-CD3, clone OKT3	American Type Culture Collection
anti-CD28, clone 10F3	Invitorgen

Human recombinant IL-2 (Tecine)	Hoffmann-Roche
Ionomycin	Sigma Aldrich
Phytohemagglutinin (PHA)	Sigma Aldrich
Phorbol myristate acetate (PMA)	Sigma Aldrich

2.1.2 FLOW CYTOMETRY

Antibodies for flow cytometry

Specificity	Fluorochrome	Clone	Company
CELL SURFACE STAINING			
Lymphocytic cell subsets			
anti-CD3	FITC	OKT3	BioLegend
anti-CD3	Brilliant Violet 421	UCHT1	BioLegend
anti-CD3	PerCPCy5.5	OKT3	BD Biosciences
anti-CD4	APCCy7	RPA-T4	BioLegend
anti-CD4	APC	SK3	BioLegend
anti-CD4	Pacific Blue	RPA-T4	BioLegend
anti-CD5	PECy5.5		
anti-CD8	FITC	RPA-T8	eBioscience
anti-CD8	V500	RPA-T8	BD Biosciences
Treg markers			
anti-CD25	PE	2A3	BD Biosciences
anti-CD25	Brilliant Violet 421	BC96	BioLegend
anti-CD39	PECy7	A1	BioLegend
anti-CD39	FITC	eBioA1	eBioscience
anti-CD127	PerCPCy5.5	HCD127	BioLegend
anti-CD127	PE	hIL-7R-M21	BD Biosciences
Activation markers			
anti-CD26	PE	M-A261	BD Biosciences
anti-CD69	FITC	L78	BD Biosciences
anti-GARP	Alexa 647	G14D9	eBioscience
anti-HLA-DR	FITC	G46-6	BD Biosciences
anti-LAP	PE	TW4-2F8	BioLegend
Ectoenzymes			
anti-CD38	Alexa 488	HIT2	BioLegend
anti-CD73	APC	AD2	BioLegend
anti-CD73	PE	AD2	BioLegend
Maturation			
anti-CD45RA	Brilliant Violet 421	H100	BioLegend
anti-CD45RA	APC	H100	BioLegend
anti-CD62L	PE	DREG-56	BD Biosciences
anti-CD31	APCCy7	WM59	BioLegend

Effector			
anti-CCR7	APC	TG8/CCR7	BioLegend
anti-CD27	APCCy7	O323	BioLegend
Chemokine receptors			
anti-CCR4	PECy7	TG6	BioLegend
anti-CCR5	PE	2D7/CCR5	BD Biosciences
anti-CCR6	PerCPCy5.5	G034E3	BioLegend
anti-CXCR3	Alexa 488	TG1/CXCR3	BioLegend
vβ Chains			
anti-TCR vb1	PE+FITC	BL37.2	Beckman Coulter
anti-TCR vb2	PE+FITC	MPB2D5	Beckman Coulter
anti-TCR vb3	FITC	CH92	Beckman Coulter
anti-TCR vb4	PE+FITC	WJF24	Beckman Coulter
anti-TCR vb5.1	PE+FITC	IMMU 157	Beckman Coulter
anti-TCR vb5.2	PE	36213	Beckman Coulter
anti-TCR vb5.3	PE	3D11	Beckman Coulter
anti-TCR vb7.1	PE+FITC	ZOE	Beckman Coulter
anti-TCR vb7.2	FITC	ZIZOU4	Beckman Coulter
anti-TCR vb8	FITC	56C5.2	Beckman Coulter
anti-TCR vb9	PE	FIN9	Beckman Coulter
anti-TCR vb11	PE	C21	Beckman Coulter
anti-TCR vb12	FITC	VER2.32.1	Beckman Coulter
anti-TCR vb13.1	PE	IMMU 222	Beckman Coulter
anti-TCR vb13.2	PE	H132	Beckman Coulter
anti-TCR vb13.6	PE+FITC	JU74.3	Beckman Coulter
anti-TCR vb14	FITC	CAS1.1.3	Beckman Coulter
anti-TCR vb16	FITC	TAMAYA1.2	Beckman Coulter
anti-TCR vb17	PE+FITC	E17.5F3.15.13	Beckman Coulter
anti-TCR vb18	PE	BA62.6	Beckman Coulter
anti-TCR vb20	FITC	ELLI1.4	Beckman Coulter
anti-TCR vb21.3	FITC	IG125	Beckman Coulter
anti-TCR vb22	PE+FITC	IMMU 546	Beckman Coulter
anti-TCR vb23	PE	AF23	Beckman Coulter
INTRACELLULAR STAINING			
anti-CTLA-4	PE	BNI-3	BD Biosciences
anti-FoxP3	Alexa 647	259D	BioLegend
anti-FoxP3	Alexa 488	259D	BioLegend
anti-Helios	FITC	22F6	BioLegend
anti-IFN-γ	FITC	4S.B3	BioLegend
anti-IFN-γ	Pacific Blue	4S.B3	BioLegend
anti-IL-17A	Alexa 647	BL168	BioLegend
anti-IL-17A	Brilliant Violet 421	BL168	BioLegend

CONTROLS	
isotype-matched monoclonal antibodies	BD Biosciences or eBioscience

Reagents for flow cytometry

Material	Company
Clean Solution	BD Biosciences
FACS buffer	0.1% BSA 0.02% NaN_3 in 1x PBS
Flow Sheath Fluid	BD Biosciences
FoxP3/Transcription Factor Staining Buffer Set	eBioscience
IC Fixation Buffer	eBioscience
Live/dead dye (Pacific Orange succinimidyl ester)	Invitrogen
Lysing Solution	BD Biosciences
Permeabilisation Buffer, 10x	eBioscience
Rinse Solution	BD Biosciences

2.1.3 ELISA

Kits & Reagents

	Company
Human IL-17A ELISA MAX™ Deluxe	BioLegend
Human IFN-γ ELISA MAX™ Standard	BioLegend
ELISA Kit for 5'-Nucleotidase, Ecto (NT5E)	Uscn Life Science Inc.
Tween-20	ICI-Americas

Buffers & Solutions

Buffer	Compounds
ELISA Wash Buffer	0.05% Tween-20 in 1x PBS
Stopping solution	2N H_2SO_4

2.1.4 DNA

Reagents & Materials

Material	Company
2-Mercaptoethanol, 50mM	Invitrogen
DNeasy Blood & Tissue Kit	Quiagen
GeneRuler, 100 bp, 1 kb	Fermentas
Isopropanol	Roth
dNTPs	Invitrogen

FastStart Taq	Roche
QIAprep Spin Gel purification kit	Qiagen
Rotisafe	Roth
Sample preparation buffer, 1x DNA loading dye	Fermentas
TAE gel running buffer, 1x TAE buffer in de-ionized H_2O	Invitrogen

Oligonucleotides (Primer)

Primer-for: ENTPD1rs107_for 5'-GTAGAGGGAGGAAATAG-3'

Primer-rev: ENTPD1rs107_rev 5'-TGGCTACTCATGCTAT-3'

2.1.5 GENERAL EQUIPMENT

Equipment	Model/Type	Company
Autoclave	Varioclave	H+P Labortechnik
Centrifuges	5810R	Eppendorf
DNA gel electrophoresis	40-0708	Peqlab biotechnology
ELISA plate reader	Wallac Victor 1420	PerkinElmar
Flow cytometer	FACS Canto II	BD Bioscience
	FACS LSRFortesa	BD Bioscience
	FACS Aria III (sorter)	BD Biosceince
Freezers -20 °C		Liebherr and Kryotec
Freezer -80 °C	905	Thermo Scientific
Freezing Container, Nalgene Cryo 1 °C		Roth
Fridges	Comfort No Frost	Liebherr
Incubator, Incusafe	MCO-2OAIC	Sanyo
Microscope		Zeiss
Microwave	M 637 EC	Miele
Multipipette, Reasearch plus	200 µl	Eppendorf
Multipipette plus	1 ml	Eppendorf
Nanodrop	2000c	Peqlab biotechnology
Neubauer cell chamber		Marienfeld
Nitrogen tank		Tec-Lab
PCR Thermocycler	T3	Biometra
pH meter		Mettler
Pipettes		Eppendorf, Gilson
Pipette boy		Eppendorf
Racks		Roth
Standard power pack P25 Biometra®	Bio105 LVD	Biomed Analytic
Sterile bank	MSC-Advantage	Thermo Scientific
Suction pump		Sevox Medizintechnik GmbH

Table centrifuge	5424R	Eppendorf
Thermoshaker with heated lid	2.0 ml	CLF, Emersacker
Transilluminator (UV)	TI 1	Biometra
Waterbath		GFL
Vortex	Gene 2	Scientific Industries

2.1.6 CONSUMABLES

Consumable	Company
96-well plate, U-bottom and flat-bottom	Greiner
Cell strainer, 70 µm	BD Falcon
Cryo tubes	Greiner
ELISA plates, Nunc MaxiSorp® flat-bottom 96 well plate	nunc
Eppendorf tubes	eppendorf
FACS tubes	Sarstedt
Falcon tubes	BD Falcon
Gloves, nitrile and latex	Supermax Glove
Hollow needle	Sarstedt
Liquid reservoir for multichannel pipettes	Roth
Petri dish	nunc
Pipette tips	Eppendorf, Sarstedt
Pipettes with tip	Greiner
Serological pipets	BD Falcon
S-Monovette, EDTA, 7.5 ml	Sarstedt
Surgical blade	B. Braun
Syringes	B. Braun
Tissue culture flasks, 50 and 250 ml	Sarstedt

2.1.7 GENERAL SOLUTIONS

Material	Company
Dulbecco's Phosphate Buffered Saline (PBS), 1x	PAA
Ethanol, ≥99,8%	Roth
Ethanol denatured	Walter-CMP

2.1.8 SOFTWARE

Software	Company
BD FACSDiva 6.2	BD Biosciences
FlowJo 9.5.3	Tree Star, Inc.
Papers2	Mekentosj
Prism 5.0b	GraphPad Software, Inc.

2.1.9 DONORS

Donors		Ethics protocol
Thymus samples	Prof. Ali Dodge-Khatami, Department of Paediatric Cardiac Surgery	PV3459
Cord blood	Prof. Petra Arck	
Peripheral blood from children	Dr. Jun Oh and Dr. Florian Brinkert; Department of Paediatrics	PV3746
Blood from infants	Dr. Chritisn Klenmann	
MBL, CLL	Dr. Mascha Binder	
Buffy coats	Blood bank UKE	

2.2 METHODS

2.2.1 DONORS

Thymus samples

Thymus tissue was provided by Prof. Ali Dodge-Khatami (Department of Paediatric Cardiac Surgery) from children that underwent cardiac surgery. The sample collection is approved by the local ethics committee (study code: PV3459; Immunregulatorische Mechanismen in Autoimmunerkrankungen: Studien zur Phänotypisierung und Funktion von regulatorischen und Effektor-Vorläuferzellen; Applicant: Eva Tolosa).

Cord blood

Umbilical cord blood samples were obtained from the maternity clinic, in collaboration with the Laboratory for Experimental Feto-Maternal Medicine and under supervision of Prof. Petra Arck (Department of Obstetrics and Fetal Medicine).

Blood samples from children

Peripheral blood from immunological healthy children was obtained from Dr. Jun Oh and Dr. Florian Brinkert (Department of Pediatrics). Study code: PV3746 (Studie der immunpathologischen Mechanismen in juveniler idiopathischer Arthritis, Applicant: Eva Tolosa). And from infants the peripheral blood was obtained from Dr. Christian Klemann (Medizinische Hochschule Hannover).

Blood samples and buffy coats from adult donors

Peripheral blood was drawn from healthy donors recruited in the UKE. Buffy coats were obtained from the Blood Bank at the UKE.

MBL and CLL patients

Blood samples of clinical MBL and CLL patients visiting the Hamburg Medical Center's outpatient unit were obtained after written informed consent as approved by the Physician's Association in Hamburg. Age-matched healthy donors (HD) served as controls.

2.2.2 METHODS IN IMMUNOLOGY

2.2.2.1 ISOLATION OF HUMAN CELLS

Isolation of peripheral blood mononuclear cells (PBMCs)

Human PBMCs from venous blood or buffy coats were isolated by gradient centrifugation using lymphocyte separation medium (LSM, PAA), for 30 min, 2000 rpm. Prior to layering it on the LSM, blood was diluted 1:2 and buffy coats 1:4 with PBS. The PBMC layer was collected and washed three times with cold PBS (10 min at 1800 rpm and two times 5 min at 1500 rpm, 4 °C). The total cell number was determined using a Neubauer counting chamber (Marienfeld), dead cells were excluded by dye exclusion method using trypan blue (Sigma Aldrich). After counting cells were immediately used for further experiments or frozen with freezing medium.

Isolation of thymocytes

Discard thymic tissue from infants undergoing corrective cardiac surgery was collected and processed immediately after. The tissue was placed in a petri dish with PBS, finely cut and the thymocytes were mechanically separated from the stroma by mashing the tissue with the embol of a syringe. Thymocytes were then washed, counted and immediately used for further experiments or frozen with freezing medium.

2.2.2.2 FLOW CYTOMETRIC ANALYSIS AND SORTING

Flow cytometry is a technique for counting and analyzing cells, by passing them in a stream of fluid trough optical detectors coupled to photomultipliers that convert the optical signal to electronic pulses, allowing multiparametric analysis of the physical and chemical properties (Herzenberg et al., 2002; Miyara et al., 2009a; Sambrook et al., 1989). If cells are labeled with fluorochrome-coupled antibodies targeting different molecules, the intensity of the fluorescence signal is proportional to the expression of such molecules.

The antibodies used in flow cytometry are conjugated to fluorescent compounds, fluorochromes. Fluorochromes absorb and emit light on characteristic spectra. Some fluorochromes can be excited by the same laser but emit at different wavelength (such

as FITC and PE; blue laser), or the fluorochromes are excited by different lasers, likewise with different emission wavelength. Electronically collected data is compensated in order to correct for overlapping emission spectra. The data is collected for each individual cell, enabling the analysis of subpopulations within the sample. For each event and cell discriminating parameters are acquired: forward scatter light (FSC), providing information on the size of the cell and side scatter light (SSC), visualizing the granularity (Figure 2.1). By the comparison of area and height parameter of FSC or SSC, it is possible to discriminate doublets from single cells. Using antibodies differentially labeled with fluorochromes, cell populations can be identified via unique cell surface proteins recognized by these antibodies. (Figure 2.1.)

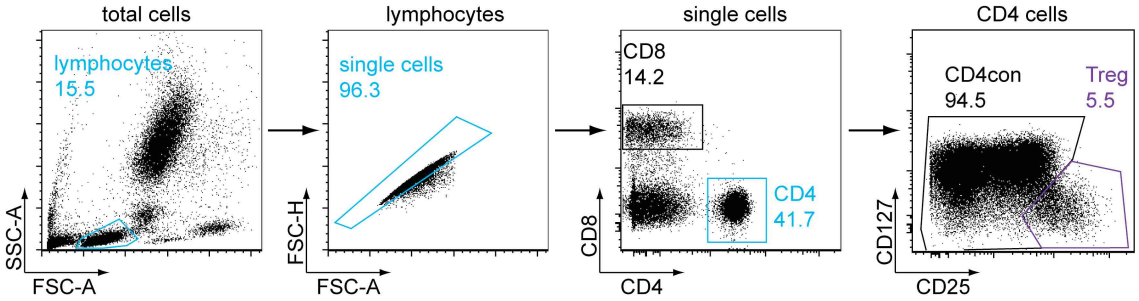


Figure 2.1 Gating strategy for regulatory T cells

The first gate is set according to the size and granularity of the cells using the FSC-A/SSC-A (A=area), here on the lymphocytes cloud. By comparing FSC-A and FSC-H (H=height) it is possible to discriminate doublets from single cells; everything that is out of the diagonal is considered as a doublet. Using fluorochrome labeled antibodies the examination of specific cell types is procurable, here gating on CD4 positive and CD8 positive cells. In the CD4 cells it is now possible to distinguish regulatory T (Treg) cells ($CD25^{hi}CD127^{lo}$, purple gate) from conventional CD4 (CD4con) cells.

In addition to surface markers, this method allows the detection of intracellular molecules such as cytokines or transcription factors. In addition to the mere analysis of cells, this method also enables to sort cells on the basis of their surface markers. During cell sorting, single cells are piped through a capillary and each cell of interest can be packed into a single drop, which is electrostatically deflected and conducted into a reaction tube or culture plate. (Galbraith, 2012; Mullis et al., 1994).

Flow cytometry was performed on BD FACS Canto II, BD LSRFortessa and for sorting BD ARIA III flow cytometer using the FACS Diva software (BD Bioscience). The data was analyzed with FlowJo software (Tree Star, Inc.).

2.2.2.3 STAINING OF SURFACE AND INTRACELLULAR MARKERS FOR FACS

For cell surface FACS analysis, cells were washed with PBS (approximately $1x10^6$ cells per staining). To block unspecific antibody binding, cells were incubated with human IgG (hIgG) for 10 min at room temperature (RT), surface antibodies diluted in FACS buffer were added and incubated for 30 min at RT in the dark. Following this, cells were washed by adding 1 ml of PBS and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 200-300 µl FACS Buffer. The cells were subsequently analyzed via FACS. In case of whole blood or cord blood, 50-100 µl of blood was blocked with hIgG and stained with surface antibodies as before. Afterwards, erythrocytes were lysed by adding 1 ml 1x Lysing Solution (BD) for 10 min at RT in the dark, followed by a washing step. Cells were resuspended in FACS Buffer and analyzed by FACS.

For the analysis of the TCR-Vβ repertoire whole blood was stained for cell surface molecules with the regulatory T (Treg) cell panel (CD3, CD4, CD127, CD25 and CD39) including the entire IOTest Beta Mark TCR-Vβ Repertoire Kit (Beckman Coulter). This kit includes a panel of Vβ family specific antibodies, which cover about 70% of normal human TCR Vβ repertoire.

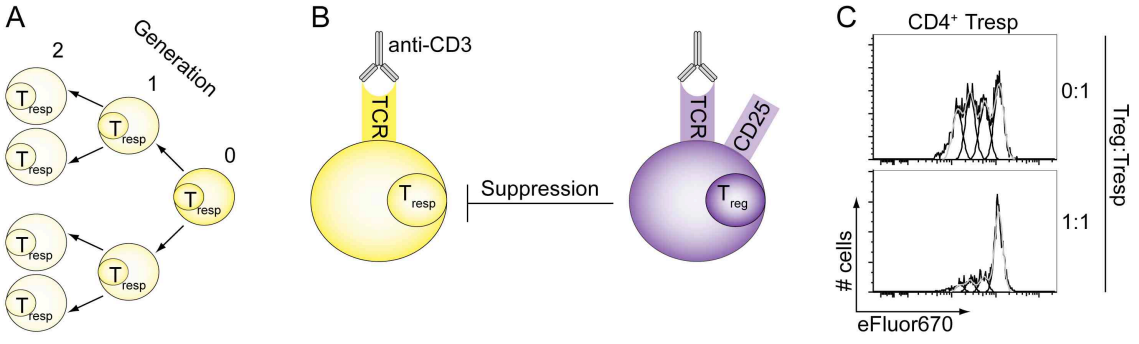
For intracellular stainings (ICS), PBMCs were first stained for cell surface markers as described above. To discriminate dead cells, an amine-reactive fluorescent dye (Pacific Orange, Invitrogen, dilution 1:1000) was added 10 min after starting cell surface marker incubation, and the incubation was proceeded for 20 min at 4 °C. Living cells react only on their cell surface with the fluorescent reactive dye (weakly fluorescent cells), dead cells with compromised membranes react with the dye throughout their intracellular content (brightly stained cells). The excess reactive dye was washed away, and cells were incubated in 1 ml fresh prepared 1x Fixation/Permeabilisation Buffer (eBioscience) for 1 hat 4 °C in the dark and washed twice with 1x Permeabilisation Buffer (PermBuffer, eBioscience). Subsequently the in PermBuffer diluted antibodies directed against transcription factors or cytokines were added and incubated for 30 min at 4 °C in the dark. After washing the cells with PermBuffer, cells were resuspended in 200-300 µl FACS Buffer and analyzed by FACS.

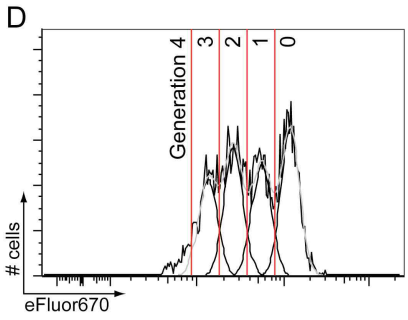
For intracellular cytokine detection, PBMCs were transferred into serum free XVIVO-15 medium (Lonza) prior to the staining and stimulated with phorbol-myristate-acetate (PMA, 50ng/ml) and Ionomycin (iono, 1 µg/ml) for 6 h at 37 °C in the incubator. To prevent the release of cytokines into the extracellular environment, Brefeldin A (10 µg/ml) was added after 1 h of incubation with PMA/iono. Brefeldin A is a substance produced by fungal organisms such as *Eupenicillium brefeldianum* and is used to block the protein transport from the endoplasmatic reticulum to the Golgi apparatus (Herzenberg et al., 2002; Marie et al., 2008; Sambrook et al., 1989). After stimulation for 6 h, cells were stained for intracellular molecules as described above. It is of note that fixed cells are not vital and cannot be used for functional assays.

2.2.2.4 TREG SUPPRESSION ASSAYS

Freshly prepared PBMCs from healthy donors were labeled with eFluor 670® (eBioscience) according to the manufacturer's protocols and used as responder cells. In brief, PBMCs were resuspended in 2 µM eFluor 670 for 10 min at 37 °C, and labeling was stopped by adding an excess of standard RPMI medium and incubation on ice for 5 min. $CD39^+$ and $CD39^-$ Treg cells or total Treg cells were FACS-sorted (gating strategy Figure 2.1) from fresh isolated PBMCs of healthy donors; MBL and CLL patients or thawed thymocytes, and added at different ratios to one hundred thousand ($1x10^5$) eFluor 670-labeled PBMC responder cells in the presence of 0.5 µg/ml soluble anti-CD3 (0.5 µg/ml, clone OKT3, BioXcell, (Figure 2.2 A, B).

For suppression assays with Treg cells from CLL patients, all patient samples were depleted from total B cells directly after PBMC isolation using the Pan T cell Isolation Kit II (Miltenyi Biotec.). The CD39 inhibitor ARL67156 (100 µM, Tocris) was added to the cultures when indicated. At days 3 or 4, supernatants were collected from the cultures, cells were stained with anti-CD4 and anti-CD8 antibodies, and eFluor 670 dilution was measured by flow cytometry (Figure 2.2 C).





Generation	% in each generation	Division number (i)	Progeny 2^i	Progenitors	% at each division
0	24	0	1	24	55,0222
1	21,3	1	2	10,65	24,4161
2	22,3	2	4	5,575	12,7812
3	21,9	3	8	2,738	6,2760
4	10,5	4	16	0,656	1,5045

Progenitors:
% in each generation / Progeny 2^i

% at each division:
(100% / sum of Progenitors) x Progenitor

% of cells in division:
100 – 55.0222 = **44.9778** (100% – undivided cells)

Figure 2.2 Suppression assay and the calculation of the parameter "% of cells in division".
(A) With eFluor 670 labeled responder T cells (Tresp) distribute the fluorescent dye during division equally to each daughter cell. (B) The activation of Tresp and Treg cells via anti-CD3 binding leads to induction of proliferation of Tresp cells, which is suppressed via Treg cell activation. (C) Illustrates the proliferation of Tresp cells in with (1:1) and without (0:1) Treg cells. (D) The analysis of the parameter "% of cells in division" was calculated in this thesis using the FlowJo software. Here is illustrated how this parameter is computed.

2.2.2.5 *IN VITRO* TRACKING OF TREG CELLS IN CULTURE

In order to track $CD39^+$ and $CD39^-$ Treg cells during *in vitro* cultures independently of CD25 expression, PBMCs were labeled with eFluor 670 as specified above, then stained for Treg cell markers, and FACS-sorted into $CD39^+$ and $CD39^-$ Treg cells. FACS-sorted eFluor 670^+ $CD39^+$ or $CD39^-$ Treg cells were added to autologous unlabeled PBMCs at a ratio of 1:20, and the cultures were stimulated with anti-CD3 (0.5 µg/ml, clone OKT3, BioXell) in standard RPMI medium (Figure 3.9). Expression of CD25, CD39, CD69, HLA-DR, CD26, CD73, and the transcription factors FoxP3 and Helios was analyzed on labeled and non-labeled cells on days 0, 2, 4 and 6.

2.2.2.6 ACTIVATION OF TREG CELLS

PBMCs were cultured with 3 µg/ml anti-CD28 (Invitrogen), 0.5 µg/ml anti-CD3 and 20 U/ml IL-2 (Hoffmann-Roche) in standard RPMI medium. After 16 h the cells were stained for Treg markers plus anti-GARP Alexa647 and anti-LAP PE, followed by an intracellular staining for FoxP3.

2.2.2.7 EXPANSION OF TREG CELLS

For expansion of Treg cell subsets, $CD39^+$ and $CD39^-$ Treg cells were FACS-sorted from total PBMCs. One hundred cells were cultured in 96 well plates with 0.1 µg/ml PHA in the presence of $2x10^5$ 40 Gray-irradiated feeder cells per well in expansion. Recombinant IL-2 (100 U/ml) was added to the cultures every 3-4 days. Cells were restimulated every 12-15 days. FACS analysis was performed on day 0 and at every restimulation for the expression of CD4, CD25, CD39, CD45RA, HLA-DR, CD73, CD62L and FoxP3.

2.2.2.8 ACTIVATION OF T CELLS FOR THE PRODUCTION OF CD73

For the activation of T cells to assess soluble released CD73, total PBMCs were isolated from blood and sorted into CD4, CD8, Treg, CD4con cells and $CD39^+$ and $CD39^-$ Treg cells. 100.000 cells of each population were cultured, in triplicates, in 96 well plates and cultured with or without anti-CD3 (0.5 µg/ml) for 24h. Supernatants were collected and stored at -20 °C for ELISA.

2.2.2.9 ELISA

Cytokine concentrations were determined in supernatants by ELISA using the human IL-17A and IFN-γ ELISA kits according to the manufacture's protocol (BioLegend).

For the detection of soluble CD73, likewise supernatants were measured using the ELISA Kit for 5'-Nucleotidase according to the manufactures protocol (Uscn Life Science Inc.).

2.2.3 METHODS IN MOLECULAR BIOLOGY

The molecular biological methods were performed, if not stated otherwise, after Sambrook et al. (Sambrook et al., 1989). All used solutions and working materials were

autoclaved or sterilized by ethanol. For all aqueous solutions de-ionized tap water was used.

2.2.3.1 PREPARATION OF DNA

DNA was isolated from whole blood using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions.

2.2.3.2 QUANTIFICATION OF DNA

The concentration of double-stranded DNA was determined spectrophotometrically by absorbance at 260 nm using the conversion factor: A_{260} = 1 = 50 µg/ml for DNA. The ratio of A_{260}/A_{280} was used as a marker for purity. Preparations with an A_{260}/A_{280} value of 1.8 – 2.0 for DNA were considered pure.

2.2.3.3 POLYMERASE CHAIN REACTION FOR GENOTYPING

The polymerase chain reaction (PCR) is a method for the *in vitro* amplification of smallest amounts of DNA (Mullis et al., 1994). Using oligonucleotides (primers) of known sequences flanking the sequence of interest. One of these primers binds to the sense strand (forward, for) and the other to the antisense strand (reverse, rev). A PCR routinely consists of denaturation, annealing and elongation steps. At first the double stranded DNA is denaturized at 90 - 95 °C. During the next annealing step, the temperature is decreased (50 - 65 °C) and the primers bind to their complementary sequences on the DNA-strands. Is the temperature increased again to 69 - 72 °C the elongation step starts, where the complementary DNA-strands are synthesized by the DNA polymerase using desoxyribonucleosidetriphosphates (dNTPs). Through cyclic repeats of these three steps the amplification of the DNA-fragments of interest increases exponentially. Here PCR reactions were used for DNA genotyping of the SNP rs10748643 in the *ENTPD1* gene.

Primer-for: ENTPD1rs107_for 5'-GTAGAGGGAGGAAATAG-3'
Primer-rev: ENTPD1rs107_rev 5'-TGGCTACTCATGCTAT-3'

Amplification of the SNP targeting region

	Volume [µl]
10x Buffer+Mg^{2+}	2
dNTPs	0.6
Primer-for	0.6
Primer-rev	0.6
DNA	3
H_2O	13
FastStart Taq Roche	0.2
	∑ =20 µl

Step	Temperature [°C]	Duration [min]
1	94	5
2	94	0.3
3	60	0.3
4	72	1
5	Step 2 to 4 repeated 10 times with a decrease of 1 °C at step 3 at each cycle	
6	94	0.3
7	50	0.3
8	72	1
9	Step 6 to 8 repeated 25 times	

2.2.3.4 AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS

PCR products were size-fractionated per agarose gel electrophoresis (Sambrook et al., 1989). Gels with 1% agarose were made in 1xTAE buffer with 5% Rotisafe (Roth). Samples were prepared with 6x loading buffer (Invitrogen) and gels routinely ran at 70 – 90 V. For documentation DNA bands were visualized by UV-illuminator and photographed. Subsequently, the fragments were cut and eluted for further processing.

2.2.3.5 AGAROSE GEL EXTRACTION OF DNA FRAGMENTS

The elution of DNA fragments from agarose gels was carried out with the QIAquick gel extraction kit (QIAGEN) following the manufacturer's protocol. DNA fragments were eluted with 25 µl de-ionized water.

2.2.3.6 DNA SEQUENCING

All DNA sequencing procedures were performed by SeqLab. The size of the PCR product divided by four corresponds to ng DNA needed for the sequencing. The required amount of PCR product plus 20 pmol of the primer-for was adjusted with de-ionized water to a total volume of 7 µl, and further processed by SeqLab.

2.2.3.7 METHYLATION ANALYSIS

DNA was isolated from FACS-sorted $CD39^+$ and $CD39^-$ Treg cells using the DNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. The concentration of isolated DNA was determined with a NanoDrop spectrophotometer (Peqlab). Approximately 500 ng genomic DNA in a volume of 50 µl was analyzed for methylation of the specific CpG moitie in the *FOXP3* locus, at Epiontis GmbH (Berlin, Germany).

2.2.4 STATISTICAL ANALYSIS

All statistic tests were performed using GraphPad Prism (version 5.0). Two-tailed unpaired Student's *t* test was used when comparing two groups, One-way ANOVA with Bonferroni post-test was used to compare multiple groups. Linear regression analysis was used to model the relationship between two parameters. Differences were considered significant if $p \leq 0.05$.

3 RESULTS

I will present the results of this thesis in three sections. First, I will point to the phenotypic and functional differences between $CD39^+$ and $CD39^-$ regulatory T (Treg) cells. Next, I will address how CD39 expression of Treg cells is regulated, and which are the functional consequences of differential expression of CD39 in Treg cells. Here, I will include insights on the ontogeny of these Treg cells. Finally, I will show which role $CD39^+$ Treg cells play in the progression from monoclonal B cell lymphocytosis (MBL) to chronic lymphocytic leukemia (CLL).

3.1 PHENOTYPE AND FUNCTION OF CD39 EXPRESSING TREG CELLS

3.1.1 MOST $CD39^+$ TREG CELLS BELONG TO THE ACTIVATED/MEMORY T CELL POOL

$CD4^+$ Treg cells differ from conventional $CD4^+$ T (CD4con) cells in their high expression of CD25 and the absence of CD127. Additionally, all Treg cells express the transcription factor forkhead box P3 (FoxP3), which is the master regulator of Treg cell development (Hori, 2003; Pesenacker et al., 2013). On the basis of these markers, we used two strategies to characterize Treg cells by flow cytometry: first, surface expression of CD127 and CD25 (Figure 3.1 A, B and C), and second, nuclear expression of FoxP3 (Figure 3.1E).

We analyzed the CD39 expression on total $CD4^+$ T cells (Figure 3.1 C, D) and found that approximately 30% of Treg cells expressed CD39. In contrast, only 3% of the CD4con cells expressed this molecule on the cell surface. Remarkably, $CD39^+$ Treg cells expressed higher levels of FoxP3 and CD25 than their $CD39^-$ counterparts (Figure 3.1 E). We measured the expression of CD39 on CD4con and Treg cells in samples from 121 donors between 0 and 69 years of age. While the expression of CD39 on CD4con cells remained always below 7%, we observed striking differences in CD39 expression on Treg cells between individuals, ranging from 2 to 70% (Figure 3.1D). In contrast, CD73, the nucleotidase that acts in concert with CD39 metabolizing AMP to adenosine, was expressed by less than 10% of both $CD39^+$ and $CD39^-$ Treg cell populations (Figure

3.2). CD4con cells expressed similar amounts of CD73 (<15%) than Treg cells, but more than two thirds of CD8 T cells expressed this molecule.

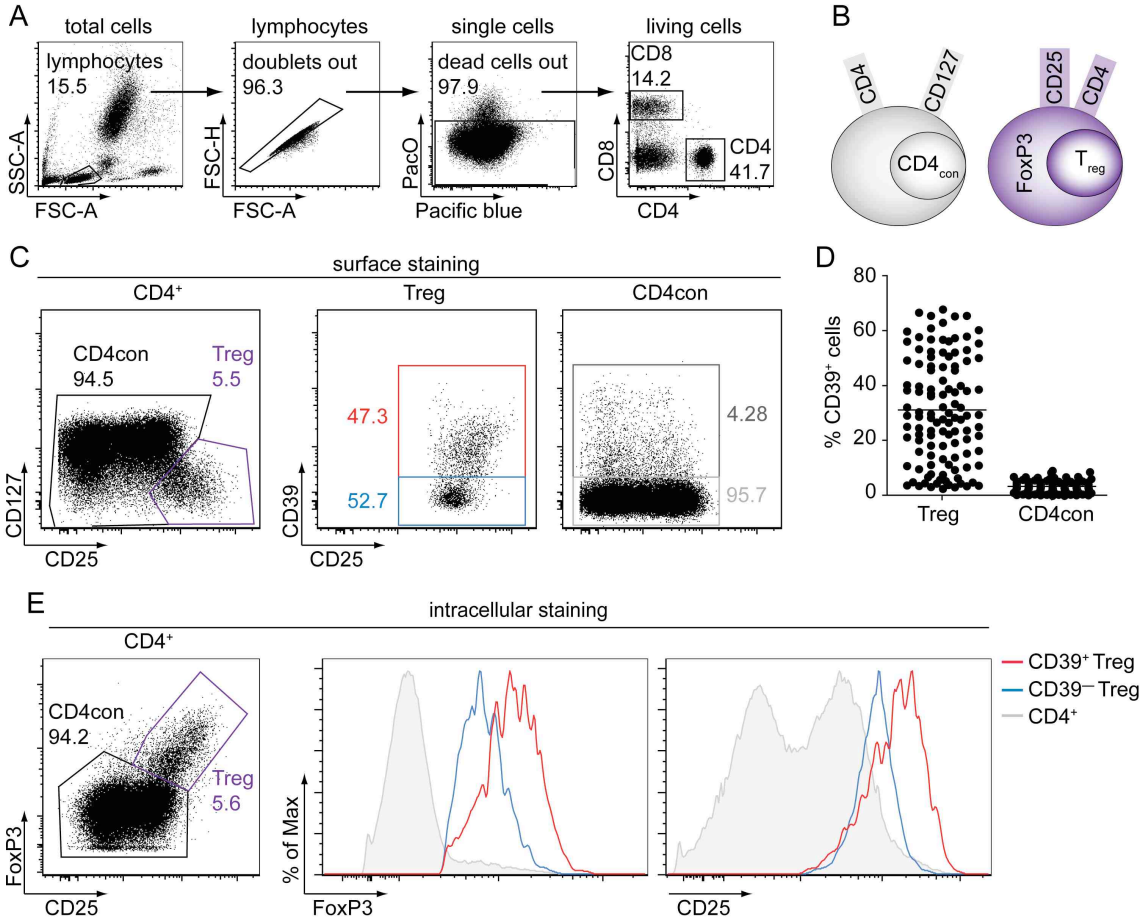


Figure 3.1. Gating strategy for Treg cells and expression of CD39.
Flow cytometry analysis of full blood or total PBMCs for the expression of CD39 on CD4 T cells. (A) Gating strategy for CD4 and CD8 T cells. (B) Scheme of the markers used for Treg cell detection: CD4con cells in black, Treg cells in purple. (C) Treg cells were gated as $CD4^+CD25^{hi}CD127^{lo}$ (purple gate), CD4con cells as $CD4^+CD25^{lo}CD127^+$ (black gate) and separated into $CD39^+$ (red gate) and $CD39^-$ Treg (blue gate) or $CD39^+$ (dark grey gate) and $CD39^-$ CD4con (light grey gate). (D) Expression of CD39 by Treg cells from healthy donors (n=121, age 0-69). (E) Intracellular staining for expression of FoxP3 and CD25 expression on $CD39^+$ (red) and $CD39^-$ Treg cells (blue). CD4con are plotted in grey.

Comparison of maturation markers on $CD39^+$ and $CD39^-$ Treg cells revealed that 85% of $CD39^+$ Treg cells displayed a central memory phenotype ($CCR7^+$ $CD27^+$ $CD45RA^-$, shown in Figure 3.2 A and B), while the $CD39^-$ Treg pool distributed between the naïve ($CCR7^-$ $CD27^-$ $CD45RA^+$) and memory compartments (Figure 3.2 A and B). HLA-DR-expressing Treg cells represent a highly suppressive Treg cell subpopulation (Baecher-Allan et al., 2006) and we found HLA-DR expression nearly exclusively on the CD39-expressing Treg cells (24%, range 15-35%). In contrast, only 3% of $CD39^-$ Treg cells

expressed HLA-DR (Figure 3.2 A and B). CD62L was similarly expressed in both populations (60%, range 45-70%). Analysis of chemokine receptors revealed that CD39$^+$ Treg cells expressed higher levels of CXCR3, CCR4, CCR5 and CCR6, which are involved in the migration of T cells to sites of inflammation (Figure 3.2 B). In contrast, we found CCR7 expressed at high levels in both subsets (Figure 3.2 B). The few CD4con cells that expressed CD39 showed a similar pattern of chemokine receptors and activation/memory markers than CD39$^+$ Treg cells (Figure 3.3).

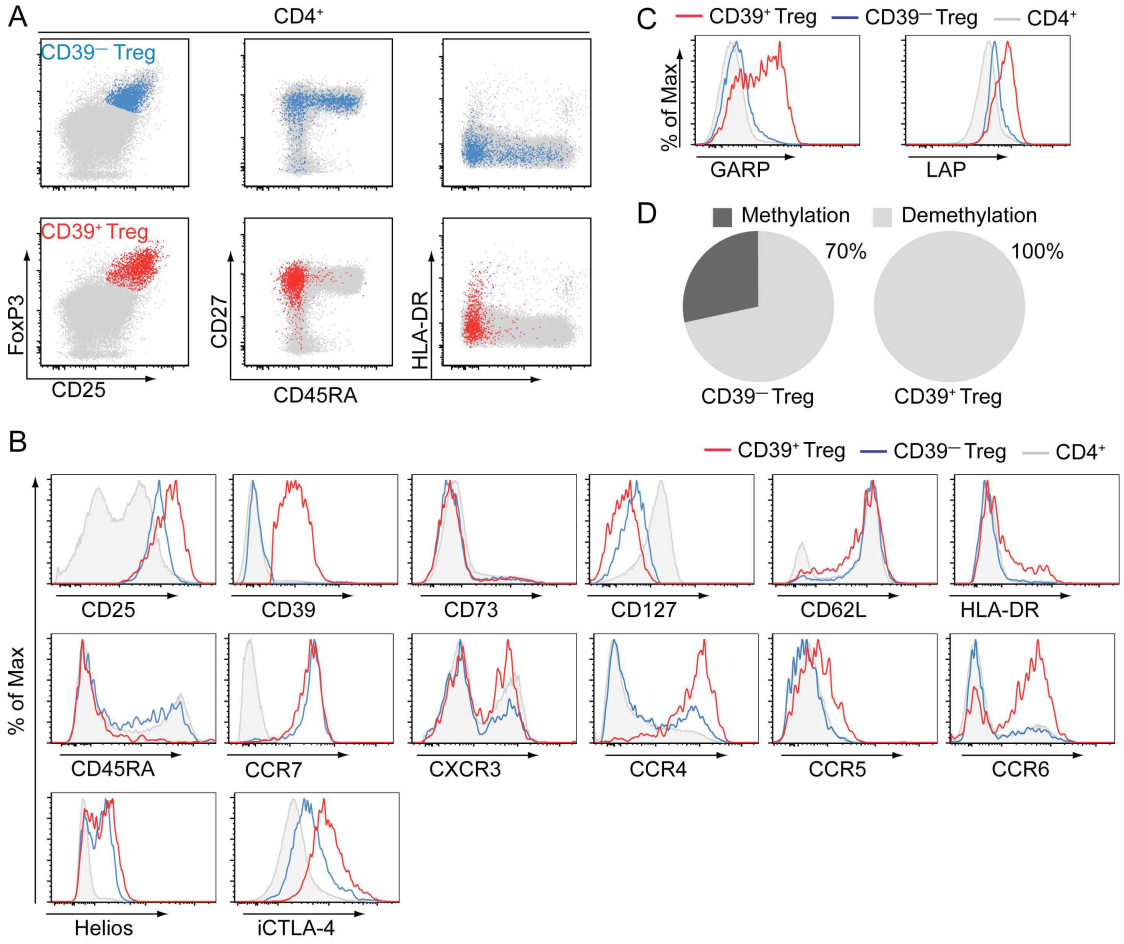


Figure 3.2 CD39$^+$ Treg cells have an activated/memory phenotype.
(A) Expression of CD25, FoxP3, CD45RA, CD27 and HLA-DR by CD39$^+$ (red) and CD39$^-$ (blue) Treg cells. (B) Expression of activation markers, chemokine receptors, ectoenzymes and transcription factors by CD39$^+$ (red) and CD39$^-$ (blue) Treg cells. (C) Expression of GARP and LAP by Treg cells after 16 h stimulation with anti-CD3/CD28. In (B) and (C) depict one representative example of four, grey plots correspond to CD4con cells. (D) CpG methylation status of FoxP3 in CD39$^+$ and CD39$^-$ Treg cells (n=3), demethylated in light grey, methylated in dark grey.

Recently, the Ikaros family member Helios was reported as a marker to discriminate thymus-derived Treg cells from peripherally derived Treg cells (Thornton et al., 2010).

Analysis of Helios expression in $CD39^+$ and $CD39^-$ Treg subsets revealed comparable expression levels on both populations: 70% (range 65-75%) of $CD39^+$ Treg cells and 60% (range 50-65%) of $CD39^-$ Treg cells expressed Helios (Figure 3.2 B). FoxP3 binding to the promoter regions of target genes mediates the developmental stability and to some extend the suppressive function of Treg cells, which leads either to increased histone acetylation (i.e. *CD25*, *CTLA-4*) and therefore increased transcription (Chen et al., 2006; Sadlon et al., 2010). The mean fluorescent intensity (MFI) of CD25 and FoxP3 was higher in $CD39^+$ Treg cells than in $CD39^-$ Treg cells (Figure 3.1 E, Figure 3.2 A). Likewise, intracellular CTLA-4 was also higher on $CD39^+$ Treg cells (Figure 3.2).

The TGF-β anchoring proteins GARP (Glycoprotein A Repetitions Predominant) and LAP (Latent Associated Peptide) are expressed only after activation on Treg cells. To analyze the expression pattern of GARP and LAP by $CD39^+$ and $CD39^-$ Treg cells, we activated PBMCs for 16 h with anti-CD3/CD28 (Figure 3.2 C). LAP and GARP expression was higher on $CD39^+$ Treg cells (65% and 85%, respectively) than in $CD39^-$ Treg cells (20% and 25%, respectively). Furthermore, epigenetic modifications of the *FOXP3* gene influence the differentiation and function of Treg cells (Maliszewski et al., 1994; Ohkura et al., 2013). We found maximal demethylation at the TSDR (Treg-specific demethylated region) of the *FOXP3* locus in the $CD39^+$ Treg cells (100%), and less (70%) in the $CD39^-$ Treg cells (Figure 3.2 D), indicating higher stability of the $CD39^+$ Treg cell linage.

In summary, the phenotype of $CD39^+$ Treg cells indicates a higher and more stable expression of *FOXP3* combined with an activated/memory phenotype.

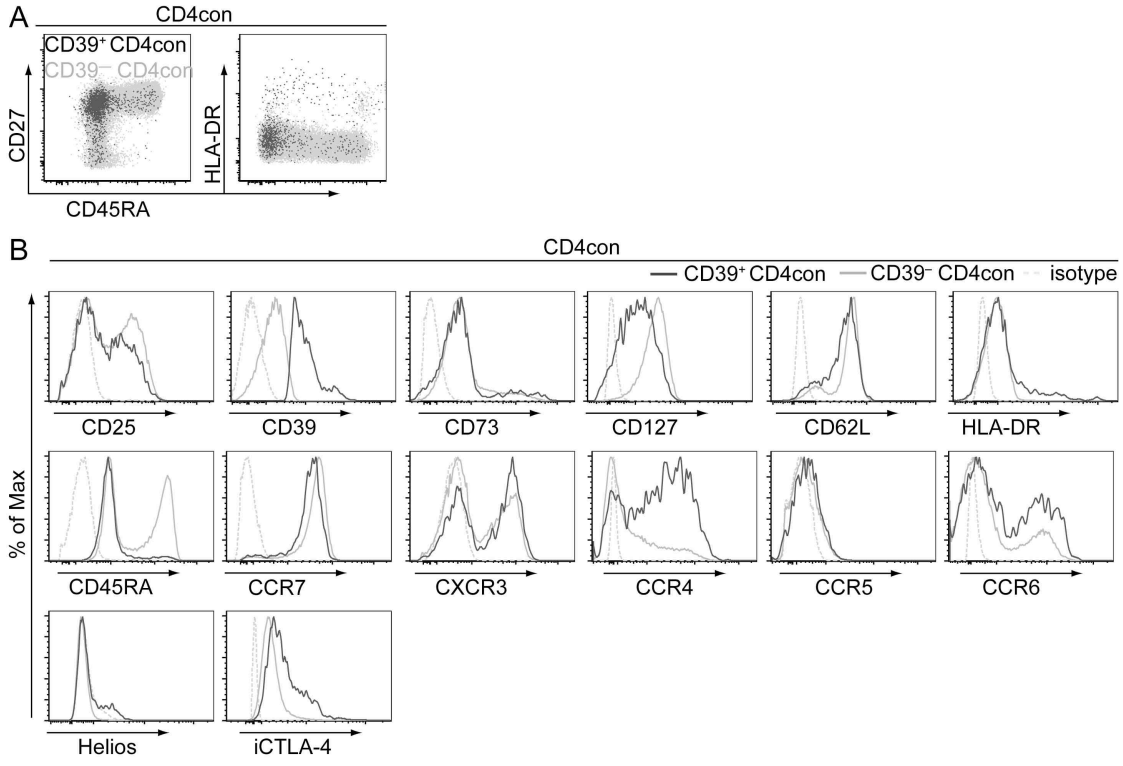


Figure 3.3 Phenotyping CD39$^+$ (dark grey) and CD39$^-$ CD4con cells (light grey) via flow cytometry. Flow cytometric analysis as in Figure 3.2. (A) Distribution of CD39$^+$ (dark grey) and CD39$^-$ (light grey) CD4con with CD45RA and CD27 or HLA-DR. (B) Expression of activation markers, chemokine receptors, ectoenzymes and transcription factors by CD39$^+$ (dark grey) and CD39$^-$ (light grey) CD4con cells, isotype control (dotted line). Representative for four individual stainings.

3.1.2 PRODUCTION OF IL-17A AND IFN-γ IS RESTRICTED TO TREG CELLS LACKING CD39

A subset of Treg cells is able to produce cytokines under inflammatory conditions (Pesenacker et al., 2013). To determine whether CD39$^+$ and CD39$^-$ Treg cells differ in their cytokine production profile we examined the *ex vivo* production of Interleukin (IL)-17A and Interferon γ (IFN-γ) by total PBMCs after PMA/ionomycin stimulation (Figure 3.4 A, B). CD4con cells produce substantially more IFN-γ (20% of the cells) than Treg cells (8%). In contrast IL-17A expression was similar in both populations (0.8%). Comparison of CD39$^+$ and CD39$^-$ Treg cells revealed that cytokine production is mostly restricted to CD39$^-$ Treg cells: While 10% of the CD39$^-$ Treg cells expressed IFN-γ and 1% IL-17A, only 1.5% of the CD39$^+$ expressed IFN-γ and 0.1% IL-17A. Surprisingly, the corresponding analysis for CD4con cells revealed that more CD39$^-$ CD4con produced IFN-γ (21% compared to 9% of the CD39$^+$ CD4con) while IL-17A was rather produced by CD39$^+$ CD4con cells (2%, versus 0.5% of the CD39$^-$ CD4con cells). Actually, a similar

frequency of IL-17A$^+$ cells was found in CD39$^-$ Treg cells and CD4con cells. Our results show that CD39$^+$ Treg cells lack pro-inflammatory cytokine production, but a large fraction of CD39$^+$ CD4con cells produce IL-17A.

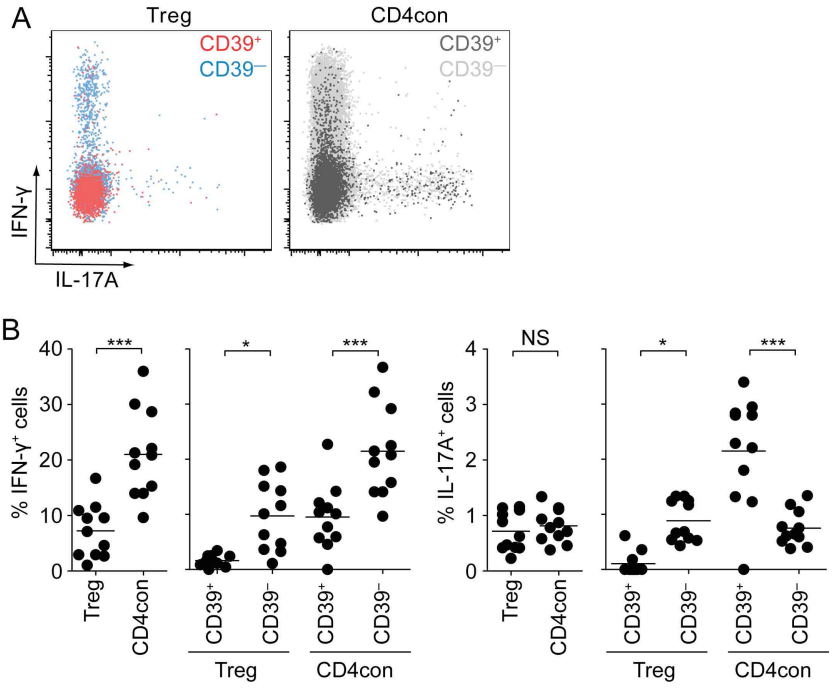


Figure 3.4 Treg cells producing IL-17A and IFN-γ do not express CD39.
PBMCs were stimulated with PMA/ionomycin and IL-17A and IFN-γ were measured on Treg cells (gated as CD4$^+$CD25^{hi}CD127^{lo}) and CD4con cells (gated as CD4$^+$CD25$^{lo/-}$CD127$^+$). (A) Representative dot plot of IL-17A and IFN-γ expression by CD39$^+$ (red), CD39$^-$ Treg cells (blue), CD39$^+$ (dark grey) and CD39$^-$ CD4con cells (light grey). (B) Percentages of cytokine-positive cells from eight donors. Horizontal bars represent means. One-way ANOVA with Bonferroni post-test, *p<0.05; **p<0.01; ***p<0.001.

3.1.3 CD39$^+$ TREG CELLS ARE EFFICIENT SUPPRESSORS OF T CELL PROLIFERATION AND CYTOKINE PRODUCTION

The capacity of Treg cells to suppress effector responses can be measured *in vitro* (Figure 2.2) (Takahashi et al., 1998; Thornton and Shevach, 1998). To compare the suppressive capacity of CD39$^+$ and CD39$^-$ Treg cells, we isolated PBMCs and stained them with CD4, CD127, CD25 and CD39 before sorting into CD39$^+$ and CD39$^-$ Treg cells. Graded quantities of these cells were then co-cultured with eFluor 670-labeled allogeneic PBMCs and stimulated with anti-CD3 (Figure 3.5). After three to four days of co-culture we collected the supernatants and analyzed the cells for the dilution of eFluor 670 in the Tresp cells (Figure 3.5). We confirmed the higher suppressive capacity of CD39+ Treg cells: at the maximum ratio of 1:1 (Treg:Tresp), CD39$^+$ Treg cells

reduced the amount of cells in division from 37.7% to 6.2%, while CD39^{-} Treg cells reduced the amount of dividing cells to 18.5% in this particular example (Figure 3.6 A). Suppression was proportional to the amount of Treg cells added to the system, however, CD39^{+} Treg cells showed a higher suppressive capacity in all assays performed. We found that Treg cells suppressed CD4^{+} Tresp cells and CD8^{+} Tresp cells in a similar fashion (Figure 3.5).

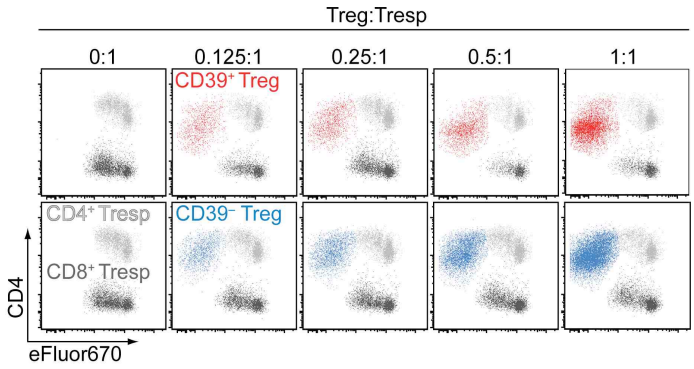


Figure 3.5 Treg cells reduce the proliferation of responder T cells in *in vitro* suppression assays.
CD39^{-} (blue) and CD39^{+} (red) Treg cells were FACS-sorted and cultured with eFluor 670-labeled PBMCs in the presence of anti-CD3. The dot plots display the proliferating CD4 Tresp cells (light grey), CD8 Tresp cells (dark grey) and the co-cultured Treg cell population.

Analysis of the supernatants revealed that CD39^{+} Treg cells suppressed IL-17A and IFN-γ production much better than their CD39^{-} counterparts, independently of the ratio of Treg to Tresp cells (Figure 3.6 B). Even with very low number of CD39^{+} Treg cells, cytokines were kept at very low levels.

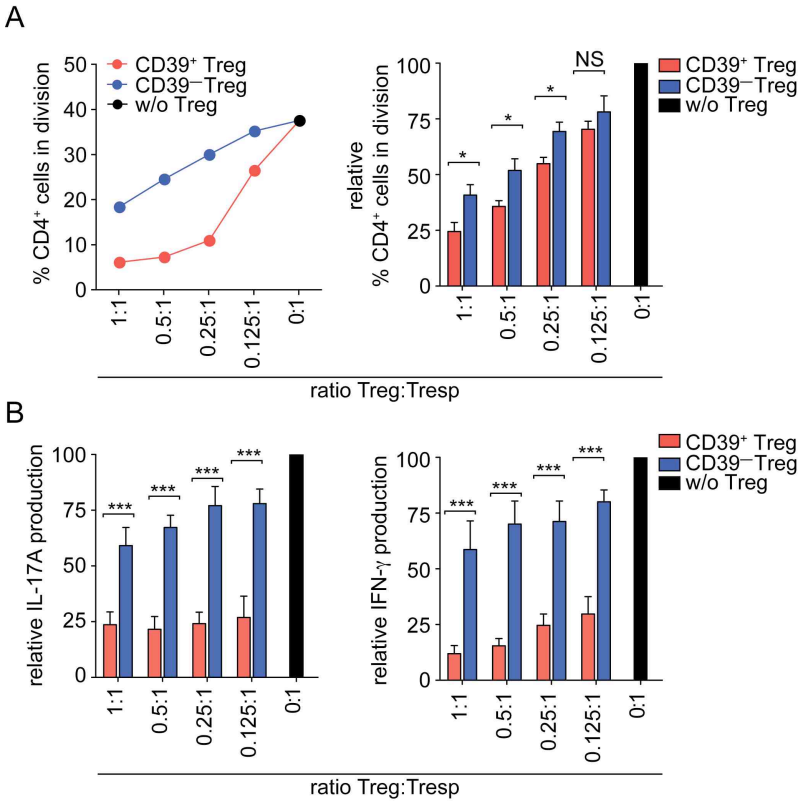


Figure 3.6 CD39-expressing Treg cells are potent suppressors.
A) FACS-sorted CD39$^+$ and CD39$^-$ Treg cells were co-cultured with eFluor 670-labeled PBMCs at different ratios and stimulated with anti-CD3. Proliferation of living CD4$^+$ eFluor 670$^+$ cells was analyzed by flow cytometry at days three or four. One representative suppression assay, left panel. The right panel represents a summary of eight independent experiments. (B) Determination of IL-17 and IFN-g in the supernatants of the experiments shown in (A). Results are shown relative to co-cultures without Treg cells (0:1). One-way ANOVA with Bonferroni post-test, *p<0.05; **p<0.01; ***p<0.001.

To further investigate the mechanistic role of CD39 in the suppressive capacity of CD39$^+$ Treg cells, we next examined the effect of adenosine on the proliferation of T cells. Adenosine is the metabolic product of the ATP-breakdown by the ectonucleotidases CD39 and CD73. For this, we added graded amounts of a metabolically stable analog of adenosine, 2-chloro-adenosine (CADO), to the proliferation cultures. After three days of culture we observed that the proliferation in response to clonotypic stimulation was reduced, in a dose-dependent manner (Figure 3.7 A). Here, 25 µM CADO were sufficient to completely block T cell proliferation. The inhibitory effect of CADO was also observed for the production of inflammatory cytokines (Figure 3.7 B).

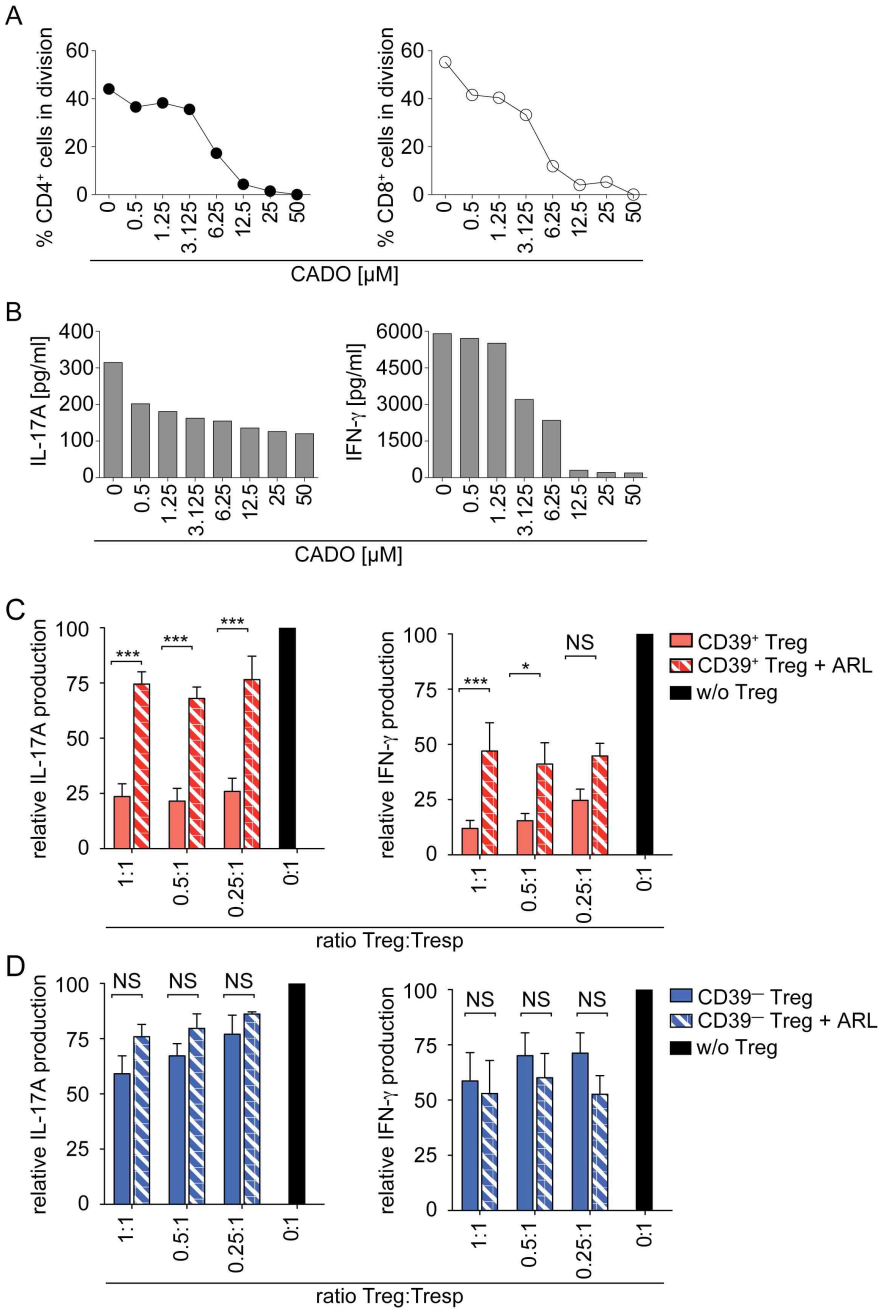


Figure 3.7 Adenosine has a suppressive effect on Tresp cells.
(A) eFluor 670-labeled PBMCs were incubated with increasing concentrations of 2-chloro-adenosine (CADO, 0-50 µM) for 3 days (1x10^5 cells/well). Proliferation of CD4$^+$ (back circles) and CD8$^+$ (empty circles) T cells was determined by flow cytometry. (B) Determination of IL-17A and IFN-g in the supernatants of this experiment. Blockade of CD39 with ARL67165 (100 µM) in suppression assays. IFN-g and IL-17A production in the supernatants is shown for co-cultures with (C) CD39$^+$ Treg cells (n=3) and (D) CD39$^-$ Treg cells (n=3). The percentage of cells in division and cytokine production were normalized to the values of effector T cells without Treg cells (0:1). Results are shown relative to co-cultures without Treg cells (0:1). One-way ANOVA with Bonferroni post-test, *p<0.05; **p<0.01; ***p<0.001.

We further analyzed the effect of blocking CD39 ectonucleotidase activity by adding the CD39 inhibitor ARL67156 to the co-cultures. Addition of the inhibitor moderately

decreased the suppressive capacity of CD39$^+$ Treg cells regarding Tresp cell proliferation (data not shown). However, CD39$^+$ Treg cell-mediated suppression of cytokine production, especially of IL-17A, was almost abolished (Figure 3.7 C). In contrast, ARL67156 had no effect on the suppression of proliferation and cytokine production by CD39$^-$ Treg cells (Figure 3.7 D). Interestingly, suppression of cytokine production by CD39$^+$ Treg cells in the presence of ARL67156 was comparable to CD39$^-$ Treg cells. This data demonstrates that adenosine production via the CD39/CD73 axis plays a key role in the suppressive action of CD39$^+$ Treg cells and indicates that the ATPase activity of CD39 is a crucial factor for the suppressive capacity of CD39$^+$ Treg cells.

3.1.4 CD73 IS PROVIDED IN A SOLUBLE FORM

The latter results imply the involvement of the CD39/CD73 axis in the generation of adenosine. In mice CD73 is co-expressed with CD39 on nearly all Treg cells (Airas et al., 1995; Deaglio et al., 2007; Friedman et al., 2009b; Hori, 2003). However, human Treg cells expressed only low levels of CD73, regardless of CD39 expression (Figure 3.2 B).

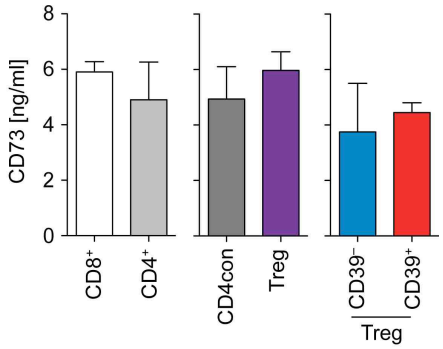


Figure 3.8 Detection of soluble CD73.
FACS-sorted CD8, CD4, CD4con, Treg and CD39$^+$ and CD39$^-$ Treg cells (100.000 cells/well) were cultured for 24 h in the presence of anti-CD3. The supernatants were collected and the amount of CD73 production was determined via ELISA.

We speculated that CD73 could be provided in soluble form rather than cell surface-bound. Sorted CD4 and CD8 cells, Treg cells, and CD39$^+$ and CD39$^-$ Treg cells were cultured for 24 h in the presence of anti-CD3, and the supernatants were tested for soluble CD73 in an ELISA. All analyzed populations produced similar amounts of soluble CD73 (in average 5 ng/ml), regardless whether the cells were stimulated with anti-CD3

or not (Figure 3.8 represents the result of stimulated cells). Thus, T cells in culture secrete CD73.

3.2 ORIGIN AND STABILITY OF CD39 EXPRESSING TREG CELLS

3.2.1 CD39 IS PARTIALLY UPREGULATED IN CD39$^-$ CELLS UPON POLYCLONAL STIMULATION

CD39 has been considered as activation marker for lymphoid cells (Maliszewski et al., 1994) and the phenotypic analysis showed higher expression of activation/memory markers in the CD39$^+$ compartment of Treg cells and CD4$^+$ T cells. To address the question whether CD39$^-$ Treg cells can convert into CD39$^+$ Treg cells upon activation, we established a culture system that enabled us to track Treg cells independently of the expression of CD25, CD127 and FoxP3, since all these markers change upon activation. For this, pre-labeled PBMCs were FACS-sorted into CD39$^+$ and CD39$^-$ Treg cells and put back into culture with unlabeled autologous PBMCs (ratio 1:20) and anti-CD3 (Figure 3.9). After six days, we analyzed the expression of makers characterizing the activation status of the cells together with Treg specific markers by flow cytometry. T cell activation results in expression of CD25 and CD69. During the days of co-culture, CD25 was upregulated in both eFluor$^-$ CD4-gated T cells and eFluor$^+$ Treg cells. Moreover, high levels of CD69 expression confirmed activation of both CD39$^+$ and CD39$^-$ Treg cells as it was the case in all T cell subpopulations (from 0% to >60%; Figure 3.10 A, B). HLA-DR was slightly upregulated in 20% of CD39$^-$ Treg cells and CD4$^+$ T cells cells after two days of activation, but at much higher levels (up to 60%) in CD39$^+$ Treg. CD39 expression on CD39$^+$ Treg cells remained high and stable over the six days of culture, yet only one third of the CD39$^-$ Treg cells and CD4$^+$ T cells upregulated CD39 upon activation (Figure 3.10 A, B). Less than 10% of both Treg cell populations expressed CD73. Stimulation led to a slightly increased frequency of CD73-expressing CD39$^+$ Treg cells (19%, range 15-24%) but did not affect the CD39$^-$ subset (Figure 3.10 A, B). In humans, the adenosine degrading enzyme adenosine deaminase (ADA) is found on the cell surface bound to CD26 (Dong et al., 1996; Schrader et al., 1990). We found that, upon activation, over 60% of CD39$^-$ Treg cells and CD4$^+$ T cells

expressed CD26. By contrast unactivated CD39$^+$ Treg cells did not express CD26 and activation induced CD26 expression in no more than 20% of CD39$^+$ Treg cells (Figure 3.10 A, B). FoxP3 expression was preserved in both CD39$^+$ and CD39$^-$ Treg cell populations, and the expression of Helios was not altered upon activation in any of the cell types analyzed.

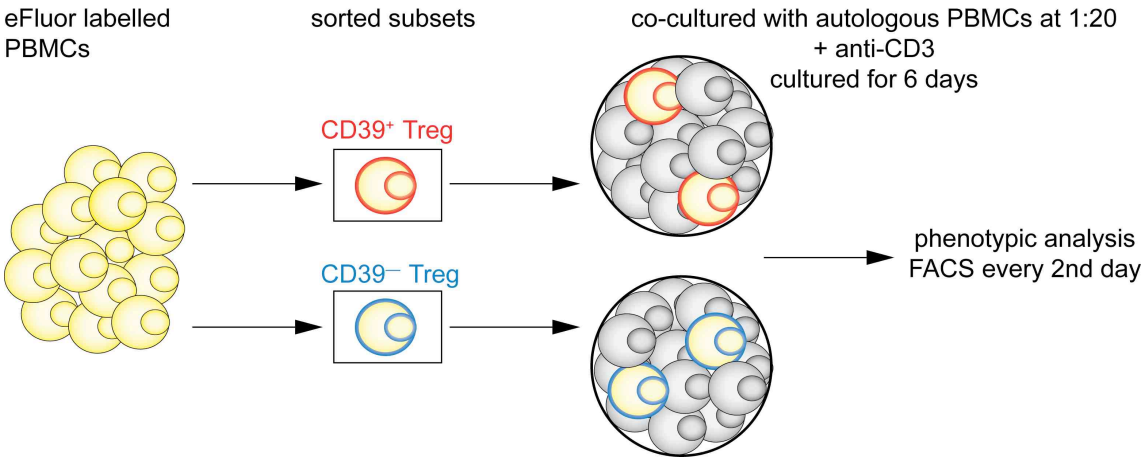


Figure 3.9 Experimental setup to monitor Treg cells during the course of activation.
eFluor 670-labeled cells were stained with Treg markers (CD4, CD25, CD127 and CD39), FACS-sorted into CD39$^+$ and CD39$^-$ Treg cells and co-cultured at a ratio of 1:20 for 6 days with autologous unlabeled cells with anti-CD3.

Six days of activation can be considered as short-term stimulation and does not resemble the stimulation environment of chronic inflammation. To mimic this situation, we expanded FACS-sorted CD39$^+$ and CD39$^-$ Treg cells by repetitive stimulation with phytohemagglutinin (PHA) in the presence of allogeneic feeder cells and recombinant interleukin 2 (IL-2) for six weeks. During this period, formerly CD39$^-$ Treg cells showed slightly elevated levels of CD39 expression (up to 40%), but the mean fluorescence intensity for CD39 expression was four-fold lower compared to the expanded CD39$^+$ Treg cells (Figure 3.10 C).

The combination of CD39 expression, together with the lack of CD26 confers CD39$^+$ Treg cells the capability to produce and sustain locally high concentrations of extracellular adenosine and exert a potent immunomodulatory effect.

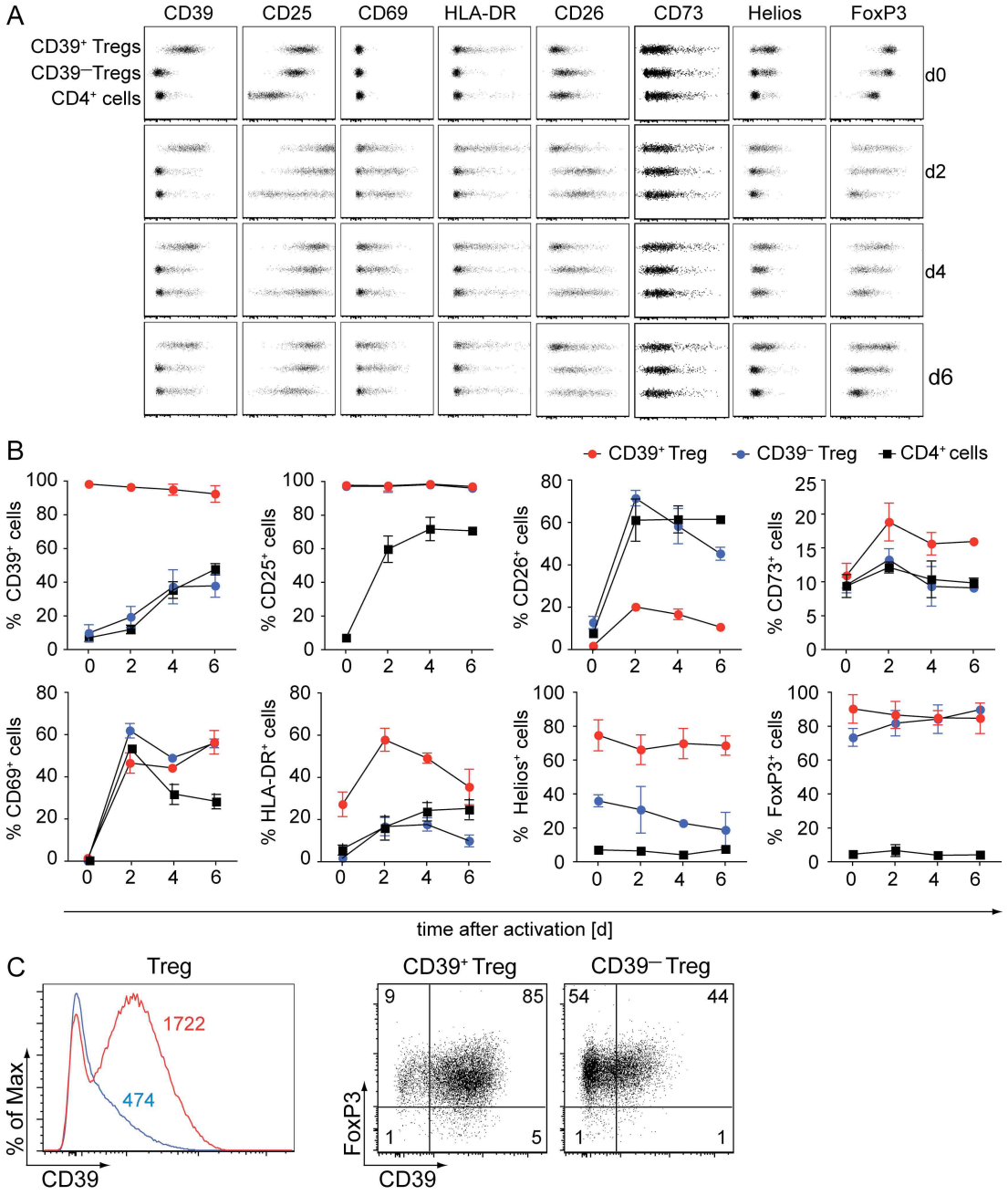


Figure 3.10 CD39 is partially upregulated on Treg cells after stimulation.
eFluor 670-labeled PBMC were FACS-sorted into $CD39^+$ and $CD39^-$ Treg cells and cultured at a ratio of 1:20 ratio with unlabeled autologous PBMC in the presence of anti-CD3. Flow cytometric analysis of the expression of CD39, CD73, activation markers and transcription factors was performed on days 0, 2, 4 and 6. (A) One representative example of Treg activation. (B) Changes in the percentage of cells expressing these markers during stimulation. Data show the average of five independent experiments. (C) Sorted $CD39^+$ and $CD39^-$ Treg cells were stimulated with low PHA and expanded in bulk for four weeks in the presence of IL-2. The graphs show the expression of CD39 in expanded cells originating from $CD39^-$ (blue) and $CD39^+$ (red) Treg cells. The numbers in the histogram indicate the mean fluorescence intensity for CD39 on gated $FoxP3^+$ cells. In the dot plots the percentage of CD39-expressing cells is shown.

3.2.2 CD39 EXPRESSION IN DIFFERENT DONORS IS HIGHLY VARIABLE BUT STABLE OVER TIME

We have seen that during the course of *in vitro* activation and also *in vitro* expansion of Treg cells, CD39 is only partially upregulated by former $CD39^{-}$ Treg cells. Considering the high inter-individual variability of $CD39^{+}$ Treg cell frequencies and the observed upregulation of CD39 by $CD39^{-}$ Treg cells after repeated stimulation, we asked whether age and immune experience could influence the frequency of $CD39^{+}$ Treg cells. We analyzed a total of 121 healthy donors between 0 and 69 years of age for $CD39^{+}$ Treg cell frequencies. Remarkably, the frequency of $CD39^{+}$ cells within the Treg cell compartment varied between 2% and 70% (Figure 3.2 D). When plotted against age, we found a weak correlation (r^2=0.2377), indicating a slight increase of $CD39^{+}$ Treg cell frequency with age (Figure 3.11 A). Still, at each age range, a high inter-individual variation could be observed. CD4con cells expressed very low levels of CD39, between 1% and 7%; nevertheless, we also observed a slight increase of $CD39^{+}$ CD4con cell frequency with age (data not shown). If CD39 expression on Treg cells were solely the result of activation, changes in the percentage of $CD39^{+}$ Treg cells in individual donors over a longer period of time would be observed. To test this, we prospectively monitored four donors with different $CD39^{+}$ Treg cell frequencies over two years. During this time period, the percentage of $CD39^{+}$ Treg cells of all individuals remained completely stable (Figure 3.11B). Furthermore, repeated immune attacks would warrant for oligoclonal activation, but comparison of TCR Vβ usage between $CD39^{+}$ and $CD39^{-}$ T cell populations revealed that neither $CD39^{+}$ nor $CD39^{-}$ Treg cells show a skewed TCR repertoire (Figure 3.11 C). These data indicate that expansion- and activation-induced changes in the frequency of $CD39^{+}$ Treg cells probably do not occur; but rather this indicates that the basal level of $CD39^{+}$ Treg cells is intrinsic to the individual.

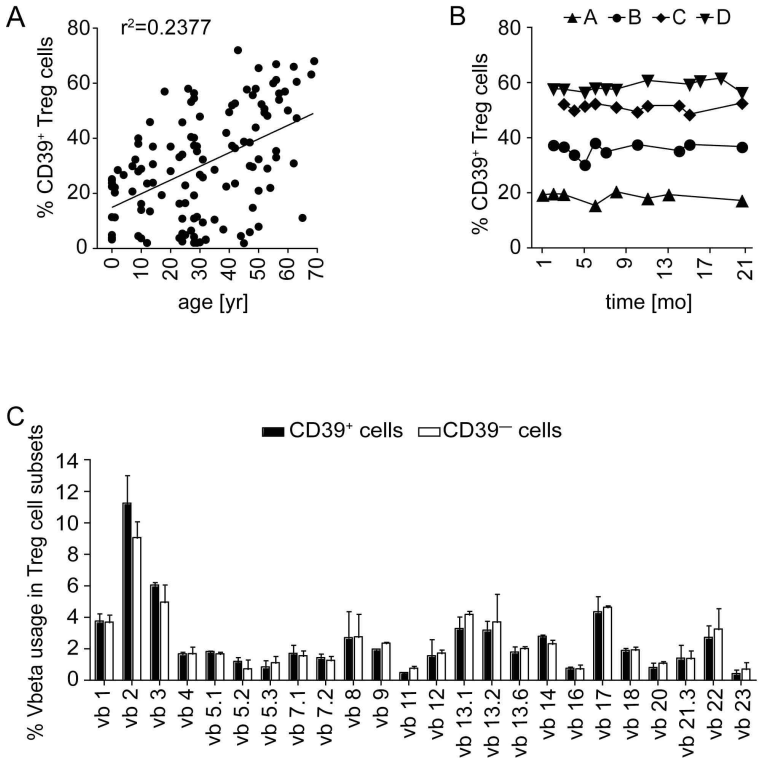


Figure 3.11 The frequency of CD39 expression in Treg cells is stable over months and increases with age

(A) Correlation of the frequency of $CD39^+$ Treg cells with age (n=121 individuals, aged 0-69). (B) Longitudinal analysis of CD39 expression on Treg cells in four donors. The graph shows the frequency of $CD39^+$ cells in the Treg compartment at different time points. (C) TCR Vb repertoire of $CD39^+$ and $CD39^-$ Treg cells. PBMCs from two donors were stained for Treg cell markers, CD39 and TCR Vβ chain antibodies. The Vβ distribution in $CD39^+$ Treg cells (closed bars) and $CD39^-$ Treg cells (open bars) is shown.

3.2.3 $CD39^+$ TREG CELLS EXIST IN EARLY COMPARTMENTS AND ARE ALREADY HIGHLY SUPPRESSIVE

Naturally occurring Treg cells develop in the thymus. The data presented so far suggest that CD39 expression on Treg cells is mostly independent of activation, so we hypothesized that CD39 expression is an intrinsic property of some Treg cells and $CD39^+$ Treg cells already exist in early immune compartments.

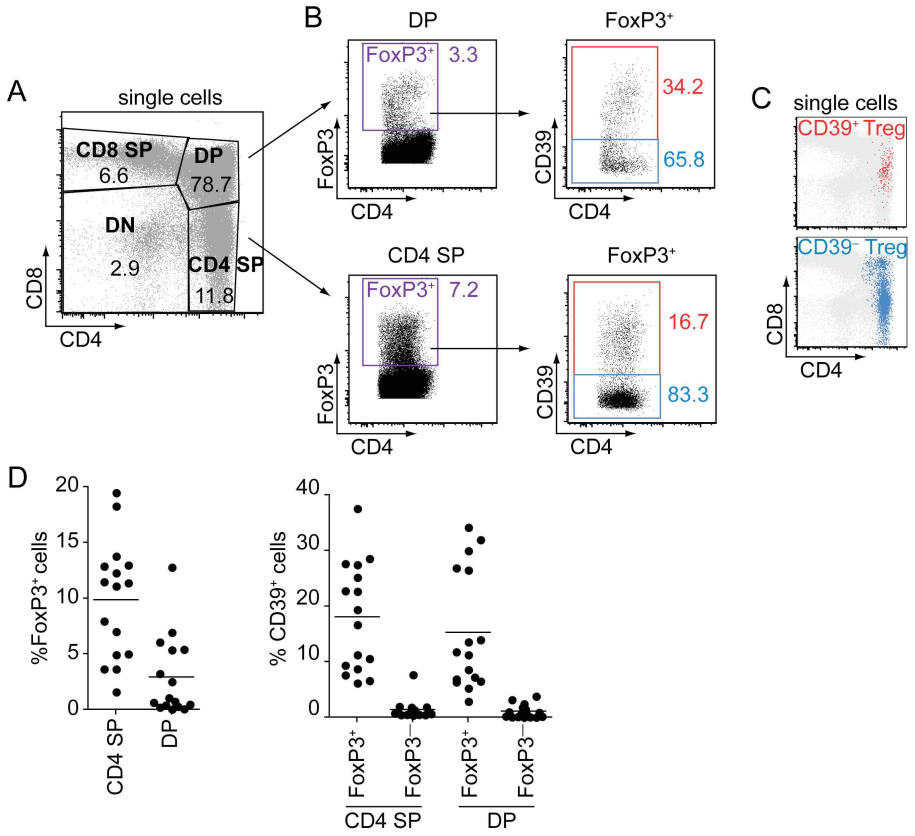


Figure 3.12 CD39$^+$ Treg cells are already present in thymocytes.
Human thymocytes were stained for CD4, CD8, FoxP3, CD25 and CD39. (A) The distribution of Treg cells using CD4 and CD8 into CD4$^-$CD8$^-$ (DN), CD4$^+$CD8$^+$ (DP), CD4$^+$CD8$^-$ (CD4 SP) and CD4$^+$CD8$^-$ (CD8 SP) is depicted. (B) The percentage of CD39$^+$ Treg cells was determined after gating on FoxP3$^+$ cells in the CD4 SP thymocytes (upper panels) and in the DP thymocytes (panels below). (C) The distribution of CD39$^+$ (red) and CD39$^-$ Treg cells (CD4 SP and DP) in the CD4/CD8 aeroplane model is depicted on top of total thymocytes (grey). (D) Frequency of FoxP3$^+$ thymocytes (left graph) and of CD39$^+$ and CD39$^-$ Treg cells (right graph) within the CD4 SP and DP thymic compartments (n=16).

To assess when CD39$^+$ Treg cells arise during the development of the immune system, we analyzed thymic tissue, cord blood and blood from infants for the presence of CD39$^+$ Treg cells. We characterized cells in different developmental stages in thymic tissue on the basis of CD4 and CD8 expression: immature CD4$^-$CD8$^-$ double negative cells (DN), CD4$^+$CD8$^+$ double positive (DP) precursor cells and CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ mature single positive cells (CD4 SP and CD8 SP), which are ready to leave the thymus (Figure 3.12 A). Flow cytometric analysis of FoxP3$^+$ thymocytes from newborns and young children revealed that an average of 18% (range 5-38%) of the CD4 SP FoxP3$^+$ Treg cells expressed CD39 (Figure 3.12 B, C, D). Interestingly, CD39 expression was detectable in the DP FoxP3$^+$ precursor cells (mean 13%, range 2-35%) (Figure 3.12 B, C,

D). This finding indicates that CD39$^+$ Treg cells develop in the thymus already as CD39-expressing Treg cells, and then migrate into the periphery.

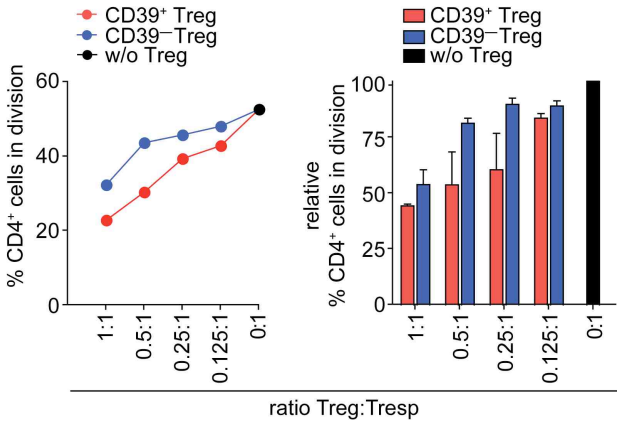


Figure 3.13 Thymic CD39$^+$ Treg cells are better suppressors than its CD39$^-$ counterparts
Sorted CD39$^+$ and CD39$^-$ thymic Treg cells (gated as CD25$^+$ in the CD4 SP compartment) were cultured with eFluor 670-labeled PBMC and stimulated with anti-CD3. Proliferation of living CD4$^+$eFluor670$^+$ cells was determined after 4 days in culture. A representative experiment is shown in the left panel and a summary of three independent experiments in the right panel. The percentage of cells in division was normalized to the proliferation of Tresp cells without Treg cells (0:1). Results are relative to co-cultures without Treg cells (0:1).

We have seen earlier that CD39$^+$ Treg cells are superior suppressors of Tresp cell proliferation. To evaluate the suppressive capacity of thymic CD39$^+$ and CD39$^-$ Treg cells, we compared these cells in suppression assays. For this, we sorted human thymocytes into CD4$^+$CD8$^-$ CD25$^+$CD39$^+$ and CD39$^-$ Treg cells and cultured them with eFluor 670-labeled allogeneic PBMCs and anti-CD3. After four days of co-culture cells were analyzed for Tresp cell proliferation (Figure 3.13). We could see that thymic CD39$^+$ Treg cells were superior suppressors than their CD39$^-$ counterparts: At the maximum ratio of 1:1 (Treg:Tresp), CD39$^+$ Treg cells reduced the percentage of cells in division from 40% to 20%, while CD39$^-$ Treg cells reduced the percentage of cells in division to 30% in this particular example (Figure 3.13). Suppression was proportional to the amount of Treg cells added to the system, but in all performed assays CD39$^+$ Treg cells showed superior suppression of Tresp cells than CD39- Treg cells.

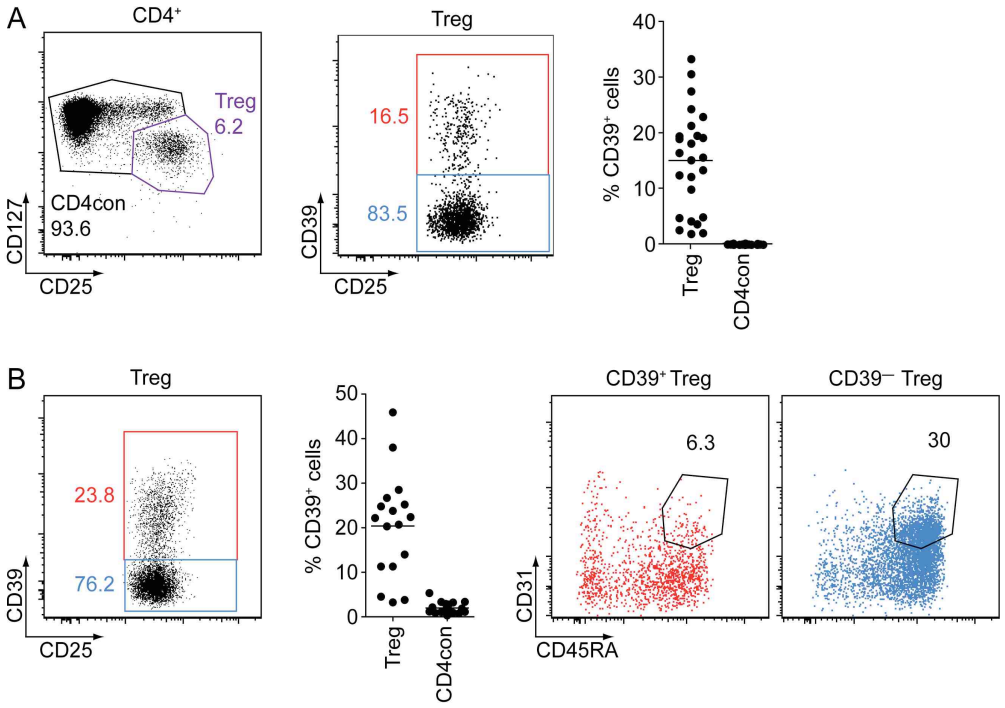


Figure 3.14 CD39$^+$ Treg cells are present in early immune compartments.
(A) Flow cytometric analysis of CD39 expression by Treg cells and CD4con cells in human cord blood (n=25), cells were stained with CD4, CD25, CD127 and CD39 (B) Whole blood from infants under one year of age was stained for Treg cells as above, and the markers CD45RA and CD31 were included to detect recent thymic emigrants (RTE), identified as CD45RA$^+$CD31^{hi}, in the CD39$^+$ (red) and CD39$^-$ (blue) Treg subsets (n=17).

The phenotypic analysis of cord blood showed that in average 15% of Treg cells (range 1-35%) expressed CD39 (Figure 3.14 A). In this case, one third of the CD39$^+$ Treg cells were naïve cells (data not shown). Furthermore, we were able to detect similar percentages of CD39$^+$ Treg cells in healthy infants under one year of age (Figure 3.14 B) and more than half of these were CD45RA$^+$. Remarkably, cells with the phenotype of recent thymic emigrants (RTE, CD45RA$^+$CD31$^+$) exist in both CD39$^+$ and CD39$^-$ Treg cell subsets, though their frequency was higher within CD39$^-$ Treg cells (20%, range 12-30%) compared to CD39$^+$ Treg cells (4%, range 2-7%; Figure 3.14 B). It was not possible to perform functional assays with cells from cord blood and blood from infants, because of limited material. The frequencies of CD39-expressing Treg cells showed already high inter-individual variability in these early compartments and were somewhat lower than later in life (Figure 3.14 A, B).

The group of Sakaguchi has proposed a new gating strategy for Treg cells, defining them on the basis of their maturation status (Miyara et al., 2009a). Using this gating

strategy CD4$^+$ T cells are characterized on the basis of their CD25 and CD45RA expression. The analyses revealed six subpopulations (Fraction, Fr. I-VI). We applied this strategy to our samples and compared peripheral blood from adults and infants. This showed that, early in life, CD39 is expressed in naïve Treg cells (Fr. I) and in mature Treg cells (Fr. II). In adults, by contrast, it is the mature Treg fraction (Fr. II) expressing CD39 (Figure 3.15) In infants as well as adults some of the cells in Fr. III, which are mature and show a low expression of CD25, express CD39. Whether these cells in Fr. III are Treg cells still is a matter of debate.

This result once more points out that Treg cells have the ability to express CD39 already early in life and that during the course of development these cells rapidly mature, supporting our hypothesis that a subset Treg cells leaves the thymus already as CD39$^+$.

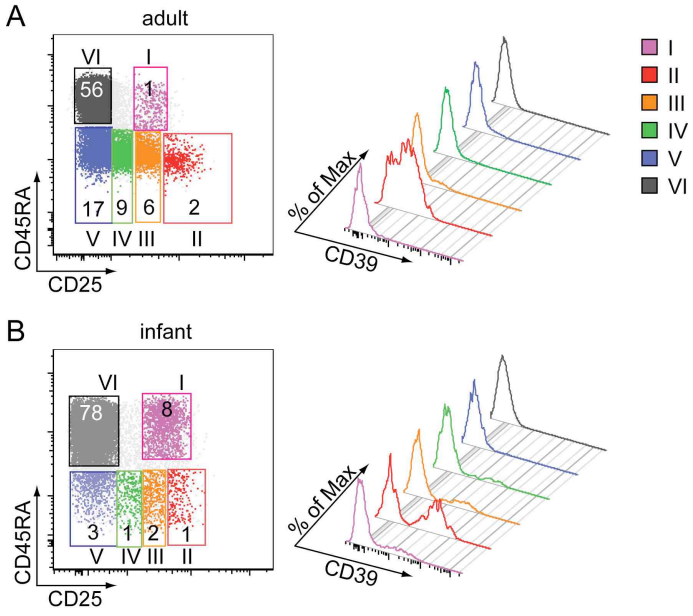


Figure 3.15 Characterization of CD4$^+$ T cells on the basis of their maturation status (according to Miyara *et al.*, 2009a)
Six subsets of CD4$^+$ T cells are defined according to the expression of CD45RA and CD25: pink line (Fraction I), CD25$^+$CD45RA$^+$ cells; red line (II), CD25^{hi}CD45RA$^-$ cells; orange line (III), CD25$^+$CD45RA$^-$ cells; green line (IV), CD25^{lo}CD45RA$^-$ cells; blue line (V), CD25$^-$CD45RA$^-$ cells; black line (VI), CD25$^-$ CD45RA$^+$ cells. The plots show one representative adult donor (A, n=10) and one infant < one year (n=17). The histograms on the right display CD39 expression levels on each fraction.

3.2.4 CD39 EXPRESSION ON TREG CELLS IS GENETICALLY DETERMINED AND EFFECTS THEIR IMMUNE REGULATIVE POWER

Our results demonstrate a high inter-individual variability in CD39 expression by Treg cells in multiple developmental stages. Friedman and colleagues recently described a Crohn's-disease-associated single nucleotide polymorphism (SNP, rs10748643) of the *ENTPD1* gene that influences CD39 mRNA expression levels in Hapmap lines (Friedman et al., 2009b). This non-synonymous SNP is located in the first intron of the *ENTPD1* gene (Figure 3.16 A). To assess whether this SNP drives the CD39 expression on Treg cells, we examined the haplotype structure across the region in 40 individuals from our cohort. In primary human immune cells we found that the presence of the different allelic variants (the rs10748643 genotype) correlated with expression levels of CD39 on Treg and CD4con cells (Figure 3.16 B): Individuals with homozygous GG showed the highest percentage of $CD39^+$ Treg cells (average 50%, range 35-60%), while homozygous AA individuals showed the lowest frequencies (average 6%, range 2-12%), and heterozygous donors were located in between (average 35%, range 19-45%). Regulation of CD39 expression by this SNP is not limited to Treg cells, since CD4con cells showed similar CD39 expression pattern in GG and AG donors (average 3%, range 1-5.5%) and significantly lower frequencies of CD39 expressing cells (average 0.8%, range 0.2-1.3%) in AA donors (Figure 3.16 B).

To determine if the allelic differences in SNP rs10748643 have functional consequences, we performed suppression assays using Treg cells from three AA and three GG donors and the allogeneic Tresp cells of the same independent donor. Total Treg cells ($CD4^+CD127^{lo}CD25^{hi}$) from donors with known genotype were FACS-sorted and co-cultured in different ratios with the eFluor 670-labeled allogeneic PBMCs in the presence of anti-CD3. In this setting, we could not observe differences in suppression of proliferation (data not shown). However, Treg cells from GG donors had a much higher capacity to suppress IL-17A and IFN-γ production compared to AA donors (Figure 3.16 C). Similar to the suppressive effects of $CD39^+$ and $CD39^-$ Treg cells, even few Treg cells (ratio 0.125:1) from GG donors could suppress cytokine production. In contrast, Treg cells from AA donors could suppress proliferation, and had a limited

suppressive capacity to the production of IL-17A and IFN-γ. This result establishes a direct relationship between genotype and regulatory function.

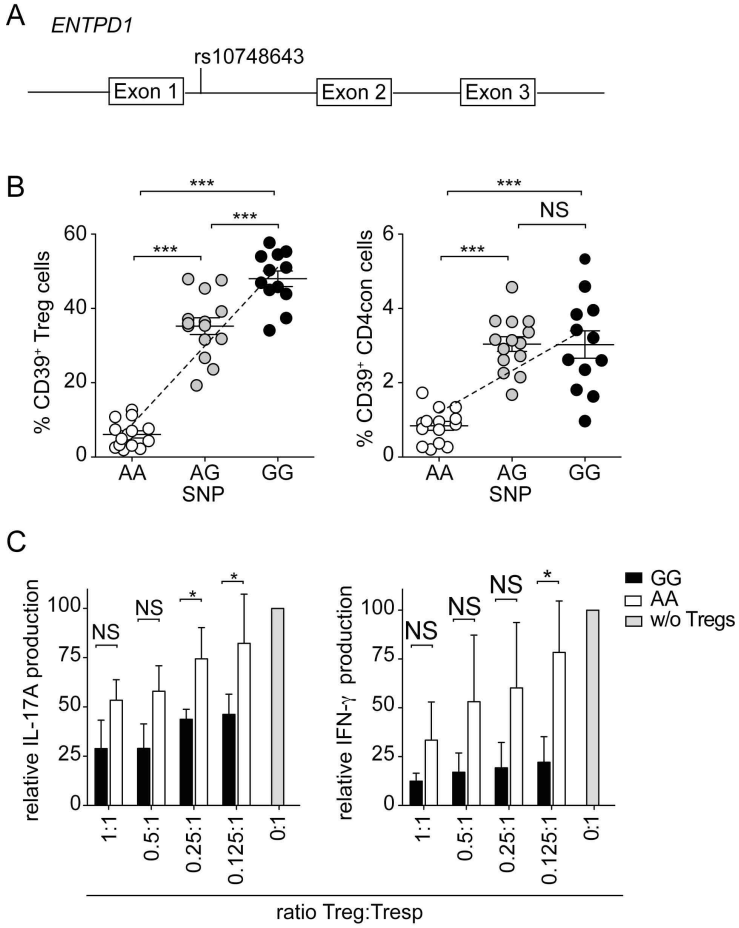


Figure 3.16 CD39 expression on Treg cells is genetically determined and has an effect on immune regulation.

(A) Locating of the single nucleotide polymorphism (SNP) rs10748643 in the CD39 gene (B) rs10748643-dependent CD39 expression in Treg (left graph) and CD4con (right graph) cells. The region comprising the SNP in the *ENTPD1* gene was sequenced and the donors were grouped according to their genotype: A/A, A/G and G/G (n=14, 14 and 12 donors, respectively). The graph shows the mean ± SEM, the linear regression (r^2=0.8341). (C) Sorted Treg cells from AA (n=3) or GG (n=3) donors were cultured at different ratios with eFluor 670-labeled PBMC of the same independent donor and stimulated with anti-CD3. Proliferation and cytokine production were analyzed as above. Results are shown relative to co-cultures without Treg cells (0:1). One-way ANOVA with Bonferroni post-test, NS=non significant, *p<0.05; **p<0.01; ***p<0.001.

3.3 IMPACT OF CD39 EXPRESSION DURING THE COURSE OF DISEASE

In the last subchapter we will evaluate if the CD39 expression by Treg cells has an impact on the progression of chronic lymphocytic leukemia (CLL). In this malignancy, Treg cells play a detrimental role by limiting the anti-tumor response. CLL is preceded by a premalignant clonal B cell expansion, termed monoclonal B cell lymphocytosis

(MBL). Which patients will progress from MBL to CLL is not predictable, and we hypothesized that CD39 expression by Treg cells might play a role.

3.3.1 SUPPRESSIVE CAPACITY OF TREG CELLS IS ENHANCED IN PATIENTS WITH MBL AND CLL

To evaluate if there are any differences regarding the CD39 expression on Treg cells between healthy donors (HD), MBL and CLL patients, we first examined total Treg cell counts (Figure 3.17 A). We found a clear expansion of Treg cells from patients with early CLL stage (95 cells/µl) compared to HD (50 cells/µl). This was even more pronounced in advanced CLL stage (250 cells/µl), while no expansion was observed in the MBL stage (50 cells/µl). When analyzing the expression of CD39 on Treg cells, we saw that the percentage of $CD39^+$ Treg cells was increased over the disease stages (HD 35%, MBL 50%, early CLL 50% and CLL 60%), however, the increase was statistically significant only in the CLL stage ($p<0.05$; Figure 3.17 B). Interestingly, despite normal Treg cell counts, the frequency of CD39-expressing Treg cells was already expanded at the MBL stage (Figure 3.17 B).

To determine if altered numbers or frequencies of $CD39^+$ Treg cells has and effect on Treg cell function, we performed suppression assays using FACS-sorted Treg cells from five HD, five MBL patients and five CLL patients and the same allogeneic Tresp cells (Figure 3.17 C). Indeed, CLL Treg cells were more effective suppressors of Tresp cells compared to HD Treg cells when the ratio of Treg cells to Tresp cells was at least 0.5:1 (Figure 3.17 D). Interestingly, MBL Treg cells also showed enhanced suppressive capacity, which appeared quantitatively intermediate between that of HD and CLL Treg cells. These experiments indicate that the alterations in regulatory function characteristic for advanced CLL initiate already at the MBL stage.

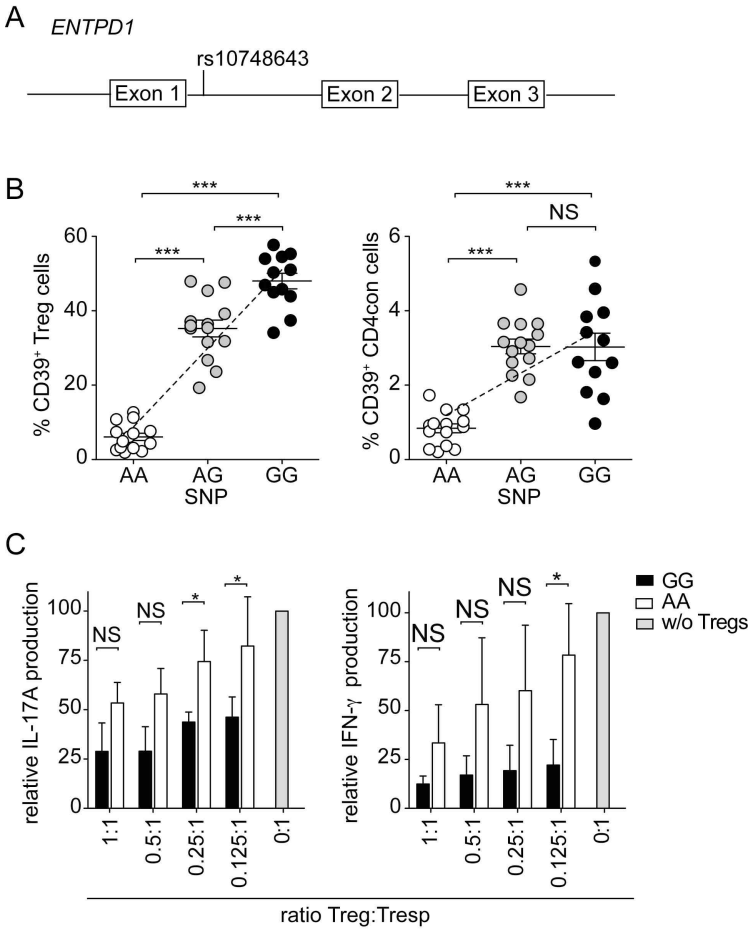


Figure 3.17 The suppressive capacity of Treg cells is enhanced in patients with MBL and CLL.
(A) Absolute Treg cell counts in HD, patients with MBL, early CLL and advanced CLL. Peripheral blood samples were stained with antibodies allowing the detection of Treg cells (CD4, CD25, CD127, CD39). (B) Percentage of $CD39^+$ Treg cells relative to total Treg cell counts in patients with MBL, early CLL and CLL. Sorted Treg cells of CLL, MBL patients or HD were cultured with eFluor 670-labeled allogeneic PBMCs HD, of the same independent donor, at different ratios in the presence of anti-CD3. Proliferation of living $CD4^+eFluor670^+$ cells was analyzed by flow cytometry at day 4. (C) Shows the suppressive effect of a 1:1 ratio of Treg to Tresp cells is shown for all donor groups. (D) Depicts the average of 5 independent donors for each group. Data show mean +/- SEM, one-way ANOVA with Bonferroni post-test; NS=non significant, *p<0.05; **p<0.01; ***p<0.001.

4 DISCUSSION

In this work I have shown that the high inter-individual variability in CD39 expression on Treg cells can be traced to a genetic single nucleotide polymorphism (SNP) located in the first intron of the *ENTPD1* gene, SNP rs10748643. Functionally, high expression of CD39 on Treg cells results in an enhanced suppressive capacity. In this section, I will discuss the functional relevance of $CD39^+$ Treg cells during the course of disease, and how the genetic determination may impinge on therapeutic approaches.

Frequency of CD39-expressing Treg cells and their functional relevance

Adenosine is a potent anti-inflammatory mediator of the immune system. CD39, acting in concert with CD73, converts the pro-inflammatory ATP to adenosine, constituting a mechanism used by Treg cells to control effector responses (Deaglio et al., 2007; Dwyer et al., 2007; Liu et al., 2006). Virtually all murine $CD4^+CD25^+$ Treg cells express CD39 and most of them co-express CD73 (Deaglio et al., 2007). In humans, by contrast, CD39 expression on Treg cells is highly variable. Regarding CD73, the enzyme required for the conversion of AMP to adenosine, its presence on the cell surface is rather modest, and it is not biased towards $CD39^+$ Treg cells, as it would be expected in order to fully metabolize ATP to adenosine. Of note, CD73 mRNA is detected in human Treg cells (Mandapathil et al., 2010). During the course of activation CD73 is only slightly upregulated on the cell surface of $CD39^+$ Treg cells, indicating that the enzymatic activity required for the conversion of AMP to adenosine in humans might be provided by CD73 in trans by neighboring cells or by soluble CD73. Indeed, the soluble form of CD73 is active (Heuts et al., 2012) and this ecto-5'-nucleotidase is readily secreted by T cells in culture. CD39 is upregulated upon stimulation, increasing the capacity to hydrolyze pro-inflammatory ATP into AMP. AMP is further degraded to anti-inflammatory adenosine by CD73, which is mainly provided as secreted protein and in trans from other cells. This leads to the hypothesis that in humans, CD39 and CD73 do not need to be co-expressed on Treg cells for the conversion of ATP to adenosine (Figure 4.1). Adenosine binds to adenosine receptors on effector cells, activates adenylyle-cyclase, and thereby increases the intracellular cyclic adenosine

monophosphate (cAMP) level (Shryock and Belardinelli, 1997). Elevated cAMP levels lead to an inhibition of proliferation and differentiation of lymphocytes and causes selective inhibition of cytokine gene expression, including IL-2 and IFN-γ. This is in part mediated by a selective suppression of the activity of nuclear factor-κB via cAMP (Minguet et al., 2005) or through the activation of the transcriptional repressor ICER (inducible cAMP early repressor) (Bodor et al., 2001). One of the suppressive mechanisms of Treg cells is to transfer cAMP into responder T cells via gap junctions (Bopp et al., 2007), without the engagement of CD39 and CD73. Treg cells, in contrast to effector T cells, seem to require high cAMP levels for mediating suppression (Whiteside et al., 2011). In Treg cells cAMP mediates the production of cyclolxygenase-2, whereby the immunosuppressive factor prostaglandin E_2 is produced, which has an inhibitory effect on effector T cells (Whiteside and Jackson, 2013).

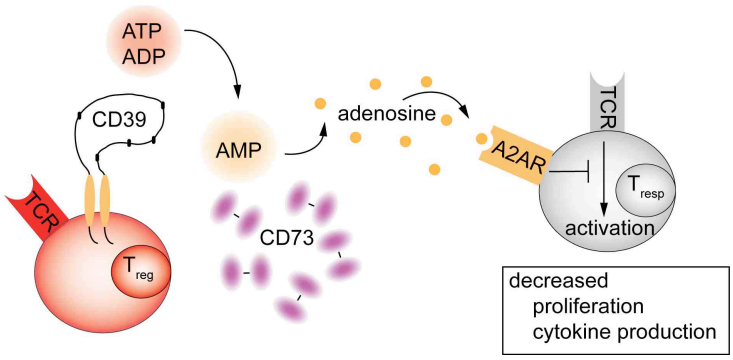


Figure 4.1 Treg cells convert proinflammatory ATP to adenosine via CD39 and CD73.
Through the expression of CD39, Treg cells are able to hydrolyze ATP and ADP to AMP. This is further degraded by soluble CD73 into adenosine, which binds to adenosine 2A receptors (A2AR) on Tresp cells and induces a downmodulation of proliferation and effector responses.

Our data confirm previous reports of higher suppressive capacity for $CD39^+$ Treg cells (Borsellino et al., 2007; Fletcher et al., 2009; Mandapathil et al., 2009). In addition, we observed that the presence of CD39 results in dramatic reduction of the production of inflammatory cytokines IL-17 and IFN-γ. The mechanism behind cytokine ablation could be adenosine production involving CD39 and CD73, followed by engagement of adenosine to its receptors on effector cells (Haskó et al., 2008) or to the destruction of local ATP, which is known to promote Th17 responses (Atarashi et al., 2008). Since pharmacological blockade of CD39 with ARL67146 rendered $CD39^+$ Treg cells

functionally close to their CD39$^-$ counterparts, we believe that adenosine production is the most likely reason for the reduction in cytokine production. Notably, CD39-deficient mice are known to have increased serum levels of TNF-α and IFN-γ (Enjyoji et al., 2008), further supporting that suppression of cytokines is achieved via the production and engagement of adenosine.

Production of IL-17 and IFN-γ within CD4 cells is associated to the effector subsets Th17 and Th1, respectively. Recently, several groups have reported that Treg cells can also produce cytokines *ex-vivo* (Ayyoub et al., 2009; Dwyer et al., 2007; Pesenacker et al., 2013). We find that only CD39$^-$ Treg cells had the ability to produce IL-17 and IFN-γ, whereas CD39$^+$ Treg cells did not. IL-17 production has been linked to Treg cells expressing CD161 (Pesenacker et al., 2013). CD161 is a marker for IL-17-producing T cells (Cosmi et al., 2008; Maggi et al., 2010), and, in addition, defines a population of cells that contain "ex-Th17" cells, former Th17 cells that have lost their capacity to produce IL-17 and gained IFN-γ production (Duhen et al., 2012; Dwyer et al., 2010; Pesenacker et al., 2013). Murine Th17 cells can be triggered *in vitro* to express CD39 and CD73, and thus acquire suppressive capacity (Chalmin et al., 2012), and have the potential to convert into Treg cells. In humans, a small proportion of conventional CD4$^+$ T (CD4con) cells express CD39. Upon stimulation with IL-2 and TGF-β, CD39$^+$ CD4con cells convert into FoxP3 expressing cells, thereby this subset serves as a reservoir of cells able to convert to Treg cells upon activation by environmental signals (Schuler et al., 2012). Previous studies have shown that peripherally derived Treg (pTreg) cells have the potential to convert into IL-17 expressing Th17 cells when stimulated with IL-6 (Xu et al., 2007). Thus, plasticity seems to be a rule, rather than an exception, of these subsets, and contributes to a quick adaptation to the changing environment during the course of an immune response.

Adenosine deaminase (ADA) is a further player in the control of pericellular levels of adenosine. ADA irreversibly degrades adenosine into inosine (Hershfield et al., 1993), which in turn can be further modified to form precursors for the production of purine nucleotides. The absence of ADA results in adenosine accumulation, leading to a severe systemic immunodeficiency (Gaspar et al., 2009; Giblett et al., 1972). In

humans, but not in mice, ADA is found on the cell surface forming a complex with two molecules of CD26 (Dong et al., 1996; Kameoka et al., 1993; Schrader et al., 1990). The functional relevance of this molecule is highlighted in the context of breast cancer, where the surface expression of CD26 is decreased in adenosine exposed tumor cells and on lymphocytes (Erić-Nikolić et al., 2011; Zhang, 2010), thus enhancing the suppression of effector T cell function. Interestingly, CD26 is present -albeit at low levels- on $CD39^{-}$ Treg cells, and is highly upregulated upon activation. In contrast, resting $CD39^{+}$ Treg cells do not express CD26, and only modestly upregulate this molecule upon activation. Thus, the combination of CD39 expression together with the lack of CD26 confers $CD39^{+}$ Treg cells the capability to produce and sustain locally high concentrations of extracellular adenosine in order to exert an immunomodulatory effect. In turn, $CD39^{-}$ Treg cells have the ability to degrade adenosine, thereby limiting its immunosuppressive effect.

A wide array of surface molecules present on subpopulations of Treg cells are associated with specific functions: HLA-DR^{+} Treg cells are highly suppressive (Baecher-Allan et al., 2006), $CCR6^{+}CD45RO^{+}$ Treg cells migrate to inflammatory sites (Kleinewietfeld, 2005), CTLA-4 contributes to the suppressor function and marks activated Treg cells (Miyara et al., 2009b; Tai et al., 2012). The $CD39^{+}$ Treg cells preferentially express all of these markers; and they showed highest expression of the Treg hallmark molecules FoxP3 and CD25. Moreover, $CD39^{+}$ Treg cells displayed a complete demethylated TSDR (Treg-specific demethylated region) at the *FOXP3* locus, which is associated with a stable and high expression of FoxP3 (Floess et al., 2007). $CD39^{-}$ Treg cells displayed a less demethylated TSDR compared to $CD39^{+}$ Treg cells. However, CD39 and CD73 are not target genes of *FOXP3* (Sadlon et al., 2010). One important question is whether CD39 itself contributes to the high suppressive capacity of $CD39^{+}$ Treg cells. The structural analogue of ATP, ARL67156, is a reliable blocker of the ectonucleotidase CD39 (Borsellino et al., 2007; Mandapathil et al., 2010; Robson et al., 2006). Specific blocking of CD39 ecto-ATPase-activity revealed an inhibition of the superior suppressive capacity of $CD39^{+}$ Treg cells. Further, blockade of the adenosine 2A receptor (A2AR) or CD73 activity results in reduced suppressive function of $CD39^{+}$

Treg cells (Alam et al., 2009; Fletcher et al., 2009; Mandapathil et al., 2010; Xu et al., 2013). In summary, the CD39/CD73-mediated production of adenosine and the binding to A2AR on effector T cells confers $CD39^+$ Treg cells an increased suppressive function compared to $CD39^-$ Treg cells. These data provide evidence that CD39 is significantly contributing to Treg cell suppressive function. Thus, CD39 marks a functionally distinct subset of Treg cells that is characterized not only by a higher suppressive capacity to block T cell proliferation, but also by the inhibition of inflammatory cytokine production.

Among $CD4^+$ T cells both CD4con and Treg cells express CD39, but only the Treg cell population mediates suppression via the generation of adenosine, whereas $CD39^+$ CD4con cells exhibit a memory phenotype and do not mediate suppression (Schuler et al., 2011; Zhou et al., 2009b). The source of CD73 is still unknown, although we have detected soluble CD73 in cultures of activated cells. While CD73 expression is low on Treg cells and CD4cons, two thirds of the $CD8^+$ T cells express CD73 (Pulte et al., 2007b), but not CD39, on their surface. In addition, CD73 is widely expressed on other cell types, including some epithelial cells and fibroblasts (Resta et al., 1998), suggesting that CD73 is not likely the rate-limiting enzyme of the CD39/CD73 axis. The two substrates for CD39, ATP and ADP, bind similarly as AMP to the active site of CD73 but cannot be hydrolyzed (Knapp et al., 2012; Sträter, 2006). The substrates of CD39 act as competitive inhibitors of CD73, thus regulating the production of adenosine.

Monocytes, neutrophils and B cells constitutively express CD39 (Koziak et al., 1999; Maliszewski et al., 1994; Pulte et al., 2007b). B cells also express high levels of CD73 (Saze et al., 2013). Endothelial cells express high levels of both ectoenzymes. CD39 and CD73 are also involved in orchestrating leukocyte trafficking in response to chemotactic stimuli (Linden, 2006; Salmi and Jalkanen, 2005), in such a way that adhesion to the endothelium is stimulated by high ATP concentrations and reduced by increasing adenosine (Salmi and Jalkanen, 2005). Since local concentrations of ATP and adenosine can be rapidly altered via the engagement of CD39/CD73, these enzymes are likely to play an important role in the pathophysiology of vascular inflammation. Indeed, mice lacking CD39 or CD73 show an increased endothelial activation,

monocyte recruitment, and platelet aggregation (Koszalka et al., 2004; Zernecke et al., 2006). Further, adenosine inhibits neutrophil adhesion to the endothelium and transendothelial migration upon engagement of neutrophil A2 receptors (Cronstein, 1994). Therefore the engagement of CD39 and CD73 protects against increased vascular permeability, which would lead to hypoxic and ischemic insults (Eltzschig et al., 2008).

While CD39 and CD73 on Treg cells contribute to the production of adenosine, the expression of CD39 on CD4con cells could limit the increasing ATP levels released from damaged cells. Elevated levels of extracellular ATP can trigger P2X receptor activation. In mice, Treg cells show an increased expression of these P2X receptors on their surface, compared to CD4con cells (Hubert et al., 2010), whereby exogenous ATP inhibits the function of Treg cells via P2X receptors (Schenk et al., 2011). ATP gates the ion channel P2X7, inducing channel opening and influx of Ca^{2+} ions (North, 2002). P2X7 has also been linked to the induction of apoptotic cell death. $CD39^+$ Treg cells are equipped to protect themselves from both, ATP-induced apoptosis and loss of suppressive function. Interestingly, $CD4^+$ T cells secrete ATP upon activation, which stimulates P2X7 in an autocrine manner and induces IL-2 gene transcription and proliferation (Yip et al., 2009), thus mediating an anti-apoptotic and proliferation-induced effect (Baricordi et al., 1999). An impairment of the CD39 expression would lead to ATP accumulation and effector T cells could increase their proliferation while Treg cells suffer apoptotic cell death. All together, CD39 controls the activity of purinergic P2X receptors and adenosine receptors by regulating extracellular nucleotide levels (Friedman et al., 2009a).

Impact of genetic determination of CD39 expression by Treg cells

CD39 has been traditionally considered as activation marker for T cells (Maliszewski et al., 1994), and our phenotypic analysis revealed that $CD39^+$ Treg cells express higher levels of activation and memory markers. How the expression of CD39 is regulated on Treg cells is not yet known.

Ontogenically, our analysis of FoxP3 expressing cells in human thymus confirmed that thymus-derived Treg (tTreg) cells exist at the $CD4^+CD8^+$ (double positive, DP) stage,

prior to the commitment to either $CD4^+$ or $CD8^+$ T cell lineage (single positive, SP) (Darrasse-Jèze et al., 2005; Nunes-Cabaço et al., 2011). In addition to the mature CD4 SP thymocytes, a fraction of DP tTreg precursor cells already expressed CD39, indicating that $CD39^+$ Treg are not just peripheral CD4 cells recirculating in the thymus, but rather thymic Treg precursors. Moreover, in early immune compartments such as cord blood, and in blood of young infants, we find $CD39^+$ Treg cells with a naïve phenotype or with phenotypical properties of recent thymic emigrants (RTE). Already in these compartments the frequencies of CD39-expressing Treg cells showed high inter-individual variability, but they were in general lower than in peripheral blood form adults. Thus, $CD39^+$ Treg cells exist within the immunologically inexperienced lymphocyte populations of cord blood, in the thymus and as RTE in periphery.

Even though CD39 is expressed on Treg cells with a high inter-individual variability, the expression of CD39 on Treg cells from one individual is very stable during the course of many months, altogether suggesting an intrinsic regulation of CD39 expression. Interestingly, the frequency of $CD39^+$ Treg cells is low at birth, but increases with age. In CD4con cells the frequency of $CD39^+$ cells also increases with age, but basal levels are much lower. This leads to the hypothesis that with successive immune reactions to pathogens or inflammatory events taking place over the years, the frequency of $CD39^+$ Treg cells increases. It remains to be investigated whether this increase is due to a higher output of $CD39^+$ tTreg cells or to the induction of $CD39^+$ pTreg cells.

A common single nucleotide polymorphism (SNP, rs10748643) in the *ENTPD1* gene tagging CD39 mRNA expression levels has been recently found associated to Crohn's disease (Friedman et al., 2009b). In Europeans, Crohn's patients are more likely to have the low CD39 expressing AA genotype, and healthy controls are more likely to have the high CD39-expressing GG genotype. In our cohort of healthy donors we could find a perfect correlation between CD39 expression of Treg cells and the SNP genotypes: AA donors showed low expression levels of CD39 on Treg cells, while donors with the GG allele showed higher levels of CD39 and a gene dosage effect was obvious with the intermediate levels displayed by heterozygous individuals. Functionally, donors carrying the AA allele harbor a decreased capacity to suppress IL-17 and IFN-γ

production by responder T cells in suppression assays when compared to GG-allele carrying donors. Further, SNP rs10748643 AA individuals show a reduced ATPase activity compared to GG individuals, confirming these functional modulations, (A. Mautner, unpublished data; Poster Moncrieffe *et al*. 2013, AAI). Independently of ethnicity, individuals with the AA allele show lower levels of CD39 protein expression (Friedman et al., 2009b), but the prevalence of the three SNP alleles (AA/AG/GG) differs among ethnic groups (see NCBI SNP database, ss17380441), with the AA allele being more prominent among Asian donors. This SNP-associated expression pattern of CD39 is not exclusive to Treg cells, since the expression of CD39 on CD4con and Treg cells correlate. Interestingly, the SNP based data revealed that donors carrying the AA allele have a similarly low expression of CD39 on CD4con cells, while AG and GG donors have a wider expression distribution. Thus, it seems that individuals with the AA allele do not have the possibility to upregulate CD39 expression. Although $CD39^{+}$ Treg cells have a higher expression level of FoxP3 as their $CD39^{-}$ counterparts, CD39 expression is not driven by *FOXP3* (Sadlon et al., 2010)..

During the course of AIDS progression, patients show a higher frequency of CD39 expressing Treg cells (Nikolova et al., 2011; Schulze Zur Wiesch et al., 2011), consistent with a downregulation of the antiviral immune response. However, Nikolova *et al.* found that out of fourteen GWAS-derived AIDS-associated SNPs non was linked to a rapid progression of the disease, but their analysis revealed that one *ENTPD1* gene variant associated with down-modulation of CD39 expression that impacts the course of disease progression (Nikolova et al., 2011). Two common polymorphisms in the *ENTPD1* gene are associated to type 2 diabetes (low CD39) and one uncommon SNP was identified to be protective (high CD39), suggesting that extracellular nucleotide metabolism may be a critical determinant of diabetes (Friedman et al., 2009a). Screening for more functional relevant polymorphisms of the *ENTPD1* gene might reveal unknown aspects of the pathophysiology of diseases involving regulation.

Relevance of CD39$^+$ Treg cells during the course of cancer, autoimmunity and AIDS

In the context of disease, CD39-deficient animals show higher susceptibility to induced colitis and fail to block allograft rejection (Deaglio et al., 2007; Friedman et al., 2009b). Treg cells play a beneficial role in EAE by suppressing inflammatory cytokine production, resulting in a decreased disease severity (Borsellino et al., 2007; Kohm et al., 2002). Interestingly, multiple sclerosis (MS) patients harbor lower frequencies of CD39$^+$ Treg cells compared to healthy donors (Borsellino et al., 2007; Fletcher et al., 2009).

In cancer, by contrast, increased immune regulation is a negative prognostic factor (D'Arena et al., 2012; Weiss et al., 2010). In the case of chronic lymphocytic leukemia (CLL), the occurrence of progression from the monoclonal B cell lymphocytosis (MBL) to early stages of CLL is low (1-2% per year) and cannot be predicted (Fazi et al., 2011). During development from healthy state to MBL and further to CLL, Treg cells increase in number, and this is accompanied by a shift towards a higher frequency of CD39 expressing Treg cells. Compared to healthy individuals, CD39 expression in peripheral blood is not only increased on Treg cells but also on all lymphocytes correlating with the stage of disease (Perry et al., 2012). Moreover, the expression of CD73 on lymphocytes is decreased in CLL patients and patients bearing CD73$^+$ clones exhibit earlier stage disease (Pulte et al., 2011). Functionally, we found that Treg cells from CLL patients had an increased suppressive capacity compared to healthy donors and MBL patients. These data had been acquired prior to the finding that the SNP rs10748643 variants correlate with CD39 expression on CD4$^+$ T cells and the suppressive capacity of Treg cells, and thus the donors were not genotyped. The excess in regulation is already seen at the MBL stage, indicating that the alterations in regulatory function characteristic of advanced CLL initiates already at the MBL stage. Nevertheless, these data need to be re-evaluated considering CD39-gene polymorphisms. Notably, other studies identified CD39-expressing CD8$^+$ regulatory T cells as highly suppressive tumor-infiltrating cells, underscoring the role of CD39-expressing Treg cells in the progression of CLL (Parodi et al., 2013).

Under steady state conditions, CD39 hydrolyzes both ATP and ADP approximately equally well, with an increased (1.5-2 fold) preference for ADP over ATP (Antonioli et al., 2013). In patients with coronary artery disease and CLL this ratio is decreased, CD39 hydrolyzes ATP and ADP with an equal preference, suggesting that inefficient or aberrant CD39 activity may be involved in the pathogenesis of arterial vascular diseases (El-Omar et al., 2005; Pulte et al., 2007a). Treg cells are sensitive to ATP-induced cell death (Aswad et al., 2005) and expression of CD39, and subsequent removal of ATP may be crucial for their immune-suppressive activity, allowing them to enter areas of inflammation and the surrounding tumor microenvironment. In cultured PBMCs from patients with CLL, ATP induces an increase in $CD4^+CD39^+$ lymphocytes (Perry et al., 2012). These data and our own data indicate that in proliferating tumor areas where cell turnover is increased, extracellular ATP promotes the induction of CD39 on $CD4^+$ cells. However, one has to keep in mind that ATP also elevates cAMP-levels in human cells, and thus delivers a potent anti-inflammatory signal itself. In human, but not murine cells, the only P2 purinergic receptor that is coupled to adenylyl-cyclase activity, P2Y11, is expressed on dendritic cells, macrophages, T lymphocytes and natural killer cells (Abbracchio et al., 2006). Therefore, released extracellular ATP from injured cells, might not solely act as an activating danger signal, but rather represent a negative feedback for immune cells to limit self-harmful effects. This mechanism suggests that the hydrolysis of extracellular ATP via $CD39^+$ Treg cells might have more complex physiologic consequences than blunt immune suppression (Gorini and la Sala, 2008). Elevated levels of this highly immunosuppressive $CD39^+$ T cell populations may support and promote the unopposed growth of CLL cells, thereby contributing to a more aggressive clinical course of the disease. In early CLL, T cells begin to severely deteriorate, coinciding with the numerical expansion of immunosuppressive Treg cells. The latter could play a role in the transition from MBL to CLL. Taken together, CD39 is a promising target for the development of novel therapies with immune modulating antitumor agents in CLL and the expression of CD73 on the malignant cell population in CLL could become a promising prognostic marker (Pulte et al., 2011).

For the development of autoimmune diseases the expression of CD39 on Treg cells is crucial. Patients with autoimmune hepatitis (AIH) (Grant et al., 2013) and MS (Borsellino et al., 2007; Fletcher et al., 2009), have lower frequencies of CD39-expressing Treg cells, concomitant with impaired suppressive function. Recovering the functionality of $CD39^+$ Treg cells in MS and AIH patients would be beneficial, enabling the efficient suppression of Th17 cells expansion, which are critically involved in the pathogenesis of these autoimmune disorders. In other autoimmune diseases, such as juvenile idiopathic arthritis (JIA) (Moncrieffe et al., 2010) and rheumatoid arthritis (RA) (Santos Jaques et al., 2013), $CD39^+$ Treg cells also show a decreased suppressive function. In contrast, increased frequencies of CD39-expressing Treg cells were found in the synovial fluid of inflamed sites, probably an attempt to control inflammation in the affected organ. Strikingly, patients with RA show an increase in their ATP/ADP metabolizing-activity and a decrease in their ADA activity (Santos Jaques et al., 2013), providing a way to understand the immune status of these patients. The impact of CD39 expression in autoimmune disorders cannot be qualified in general but needs to be analyzed individually for each disorder.

In conclusion, our study shows that the expression of CD39 in Treg cells is genetically driven and can be further modulated by immunological events over a lifetime. The regulation of ATP and adenosine levels is an important feature of the immune system and a putative target for therapy. Since immune regulation must be increased or decreased according to the type of disease, understanding the regulation of CD39 expression is a crucial first step for setting the basis for therapy.

4.1 PERSPECTIVES

For the autoimmune disease MS it was reported that $CD39^+$ Treg cells show an impaired function in suppressing Th17 cells. In contrast, an enhanced and thereby detrimental suppressive capacity of $CD39^+$ Treg cells was shown during the course of malignant diseases and AIDS. However, up to now the regulation of CD39 expression has been unclear.

The finding that CD39 expression is primarily genetically regulated pleads for the re-evaluation of the data on CD39 expression and function in the context of disease. It also prompts the question if the expression of CD39 on constitutively CD39-expressing cells i.e. monocytes, neutrophiles or B cells is also influenced by the SNP rs10748643 and further, if this has an impact on the function of these cells. Moreover, understanding the regulation of adenosine production could constitute the basis for new therapeutic strategies to restore immune regulation in MS patients or effector responses in malignant and viral diseases.

5 ABBREVIATIONS

A2AR	adenosine A2 receptor
ADA	adenosine deaminase
APC	antigen presenting cell
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
ADP	adenosine diphate
AMP	adenosine monosphate
CADO	2-chloro-adenosine
CD4con	CD4 conventional T
CLL	chronic lymphocytic leukemia
CNS2	conserved noncoding sequence 2
γ_c	common γ-chain
DCs	dendritic cells
DMSO	Dimethylsulfoxid
dNTPs	desoxyribonucleosidetriphosphates
DN	double negative
DP	double positive
ELISA	enzyme-linked immunosorbent assays
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1
FBS	fetal bovine serum
FSC	forward scatter light
FoxP3	forkhead box P3
GARP	Glycoprotein A Repetitions Predominant
GWAS	genome wide association studies
HD	healthy donors
HIV	human immunodeficiency virus
hIgG	human immune globuline G
hSerum	human type AB serum
IFN	interferon
ICS	intracellular stainings
IL	interleukin
IL-2R	IL-2 receptor
IL-7R	IL-7 receptor
iono	Ionomycin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
JIA	juvenile idiopathic arthritis
LAP	latency-associated peptide
LSM	lymphocyte separation medium
MBL	monoclonal B cell lymphocytosis
MFI	mean fluorescent intensity

MHC	Major Histocompatibility Complex
MS	multiple sclerosis
NT5E	ecto-5'-nucleotidase
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
PRRs	pattern-recognition receptors
RA	rheumatoid arthritis
ROR	retinoic orphan receptor
RT	room temperture
RTE	recent thymic emigrants
SNP	single nucleotide polymorphism
SSC	side scatter light
SP	single positive
TCR	T cell receptor
cTEC	cortical thymic epithelial cells
mTECs	medulary thymic epithelial cells
TGF	tumor growth factor
Th	T helper
Tresp	responder T
Tr1	type 1 regulatory
Treg	regulatory T
pTreg	peripherally derived Treg
tTreg	thymus-derived Treg

6 LIST OF FIGURES

7 REFERENCES

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8 APPENDIX

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Publications

1. **Anne Rissiek**, Isabell Baumann, Angelica Cuapio, Manuela Kolster, Ali Dodge-Khatami, Petra C. Arck, Friedrich Koch-Nolte, Friedrich Haag, Eva Tolosa. **2013**. A polymorphism in the CD39 gene determines expression levels in regulatory T cells and impinges on their function.

Submitted

2. **Anne Rissiek**, Ulrike Bacher, Aneta Schieferdecker, Christian Schulze, Anita Jacholkowski, Christiane Horn, Friedrich Haag, Gisa Tiegs, Martin Trepel, Eva Tolosa, and Mascha Binder. **2013**. Increasingly Suppressive T Cell Fingerprint in the Progression from Monoclonal B Cell Lymphocytosis to Chronic Lymphocytic Leukemia.

Submitted

Posters and Talks

2013	Milan, Italy ICI2013, 15th international congress of immunology Poster: *CD39 expression on Treg cells is genetically determined and can be further modulated upon activation*
2013	Schmöckwitz, Berlin 7th German Meeting on Immune Regulation Talk: *CD39 on Tregs is primarily genetically determined and is important for suppression of cytokine production*
2011	Schmöckwitz, Berlin 5th German Meeting on Immune Regulation Talk: *Origin and properties of human CD39$^+$ regulatory T cells*

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14. Oktober 2013

Sehr geehrte Damen und Herrn,

hiermit bestätige ich, dass die von Frau Anne Rissiek mit dem Titel “A single nucleotide polymorphism in the *ENTPD1* gene determines CD39 expression levels in regulatory T cells and impinges on their function ” vorgelegte Doktorarbeit in korrektem Englisch geschrieben ist.

Mit freundlichen Grüßen,

Dr. Carol Stocking

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, den 13.12.2013

Anne Rissiek