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In-depth characterization of early stages of human induced regulatory T cell differentiation by mass cytometry

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Regulatory T cells (Tregs) are responsible for the maintenance of immunological homeostasis and self-tolerance. They are part of the $CD4^+$ T helper (Th) cell population, but unlike other Th cells Tregs suppress immune responses by inhibiting other immune system cell responses, and function of antigen presenting cells. Tregs mediate suppression via contact dependent and humoral factor-mediated mechanisms. However, abnormalities in Treg numbers, frequencies, and suppressive function can trigger autoimmune diseases. Several therapies are under investigation to restore and enhance Tregs cell function in autoimmune diseases *in vivo*. On the other hand, highly activated Tregs can suppress antitumor responses, promoting cancer progression. In cancer, means to inhibit Treg function provide promising targets to control tumor cell growth.

The aim of the current study was to perform an in-depth characterization of *in vitro* generated human induced Treg (iTreg) cell differentiation at early timepoints by high-dimensional single-cell mass cytometry. For this purpose, a panel of 25 markers was designed and validated in iTregs, differentiated *in vitro* from naïve human umbilical cord blood derived $CD4^+$ T cells. The expression of these markers was further studied in iTregs compared to activated control Th0 cells over time. Additional western blot and flow cytometry analyses were performed to confirm the successful Treg differentiation by determining the Foxp3 expression.

The results show an upregulation of key transcription factor Foxp3 and several co-inhibitory molecules including PD-1, CTLA-4, LAG-3 and TIM-3 were expressed and increased with time on iTregs compared to Th0 cells. In addition, surface markers like CD103, CD137, CCR4 and CXCR3, which are interesting targets in context of Treg function and diseases, showed a statistically significant upregulation on iTregs.

In conclusion, this study gives insights in the regulation and cell surface marker expression of human Tregs at single cell level and opens new way to study Treg function.

Key words: Regulatory T cell, mass cytometry, marker.

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1 Introduction

1.1 Human immune system

The human immune system is complex and well refined system. It consists of two different parts: innate immunity and adaptive immunity; both are necessary, since they are designed to work in different situations but still in tight cooperation with each other (Marshall et al., 2018; Turvey and Broide, 2010; Bonilla and Oettgen, 2010).

Innate immunity is the initial immune defence fighting against unknown material and pathogens. Its immune responses do not require development phases or genetic recombination to mediate the function. This first line defence towards unknown pathogens occurs immediately or within hours after recognizing foreign material and acts in an antigen-independent, non-specific, manner (Turvey and Broide, 2010).

Innate immunity system has a limited repertoire of receptors, the pattern recognition receptors (PRRs), that can detect foreign materials and pathogens. The system compensates this by targeting the conserved components of microbes, pathogen associated molecular patterns (PAMPs), that different pathogens share. The cells of innate immunity are both hematopoietic (e.g., macrophages, mast cells, eosinophils, neutrophils, dendritic cells (DCs), natural killer (NK) cells) and non-hematopoietic (e.g., epithelial cells) origin. Innate immunity has also humoral components like complement proteins, C-reactive proteins, and anti-microbial peptides (Marshall et al., 2018; Turvey and Broide, 2010).

Unlike innate immunity, adaptive immunity is antigen-specific and antigen-dependent system which has also the capacity for memory through long-living memory T and B cells (Marshall et al., 2018). The key element of adaptive immunity is the tight interplay between antigen-presenting cells (APCs) and B and T lymphocytes, which facilitates the pathogen specific recognition of immunological reactions (Bonilla and Oettgen, 2010). There is a lag between the antigen exposure and the maximal response in adaptive immunity because of the antigen-specific pathogen recognition, but the response can be produced to novel and more specific targets than in innate immunity (Marshall et al., 2018; Bonilla and Oettgen, 2010).

The most important function of adaptive immunity is to recognise the certain nonself antigens, generate a pathogen specific immunologic pathway to eliminate the pathogens and develop an immunologic memory towards that specific pathogen/antigen. This happens through antigen-

specific T cells, which are activated to proliferate and antibody-producing plasma cells differentiated from B cells (Marshall et al., 2018).

Defects or dysregulations in either innate or adaptive immune system can lead to different immunopathological disorders (Marshall et al., 2018). These disorders include autoimmune diseases, hypersensitive reactions, and immunodeficiencies.

1.2 Lymphocytes

Lymphocytes are one of the central elements of adaptive immunity and the immune system of humans and other vertebrates. Humoral and cell-mediated immune responses are carried out by the two subtypes of lymphocytes B and T cells. They are responsible for structuring long-lasting and highly specific immune responses towards detected pathogens. (Turvey and Broide, 2010; Kumar et al., 2018)

1.2.1 Lymphocyte development

T cells or T-lymphocytes derive from progenitor cells in the bone marrow that migrate to the thymus for maturation and selection (Kumar et al., 2018). During the maturation, each T cell acquires own unique T cell receptor (TCR) through gene rearrangements. The TCR provides specific recognition ability to the cell by specifically recognizing the foreign antigens presented to it as peptides (Fabbri et al., 2003; Marshall et al., 2018). These peptides are presented via major histocompatibility complex (MHC) molecules which are expressed on the APCs. MHC molecules are classified into class I or class II (Marshall et al., 2018). T cells undergo positive and negative selection (Fabbri et al., 2003). In positive selection, T cells able to interact with self MHC are kept, while others will be eliminated. T cells unable to distinct self-antigens from non-self-antigens are eliminated in negative selection step, because of their reactivity to self-antigens. After selections, the T cells will be subsequently exported to periphery.

In order to get activated, T cells need to encounter APC that carry the right antigen peptide in its MHC complex. Each T cell has a co-receptor which is either cluster of differentiation (CD) 4 or CD8. T cells expressing the CD4 membrane co-receptor, $CD4^+$ T cells, can only recognize MHC class II presented exogenous peptides, while the $CD8^+$ T cells recognize only MHC class I presented exogenous peptides (Fabbri et al., 2003). Once activated $CD4^+$ or $CD8^+$ T cells are able to proliferate and differentiate into memory or effector T cells (Kumar et al., 2018; Fabbri et al., 2003).

1.2.2 CD4⁺ T helper cell populations

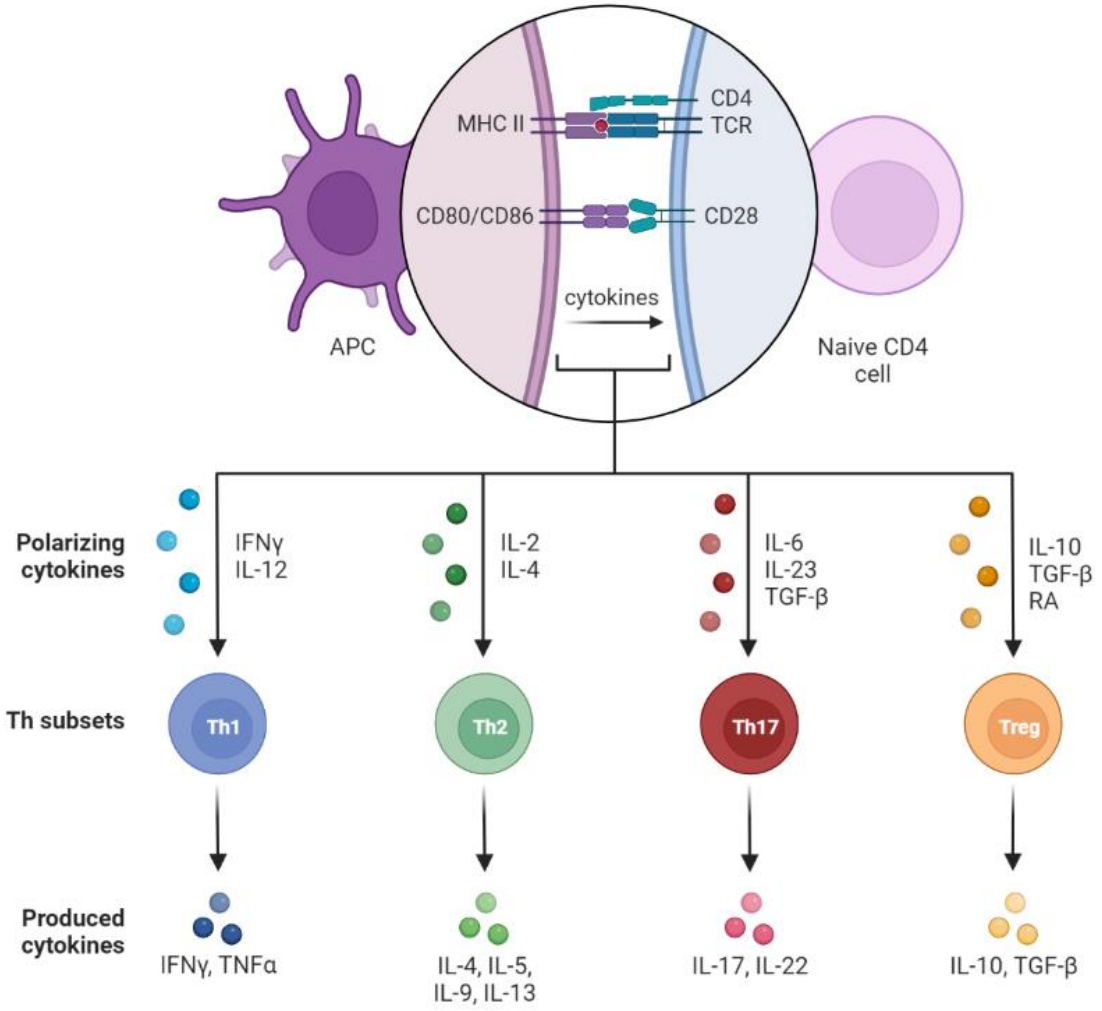


Figure 1. CD4⁺ T helper cell subpopulations. CD4⁺ T cells require three activating signals to be activated and differentiated: a T cell receptor (TCR) signal, co-stimulation (CD28) and polarizing cytokines. Specific cytokines polarize the CD4+ T cells into different subsets: T helper 1 (Th1), Th2, Th17 and regulatory T cells (Treg). These T helper subsets acquire their characteristic features. Modified from Biorender.com templates with Biorender.com.

CD4⁺ T helper (Th) cells have no cytotoxic activity, but they can mediate the immune responses by directing other immune cells to perform immune responses and pathogen clearance through releasing cytokines that influence the activity of the other cells (figure 1). Most frequent Th cell types are Th1, Th2 and Th17, but also Th9 and Th25 are part of the helper cells.

Th1 cells produce IFN-γ and interleukin (IL) -2 cytokines and contribute to microbe killing and enhancement of anti-viral immunity. Inappropriate Th1 cell responses are associated with autoimmune diseases. Th2 cell response, on the other hand, is characterized by IL-4, -5, -10, and -13 release and they drive the production of antibodies and clearance of parasites.

Imbalances in Th2 cytokine production are associated with the development of atopic conditions. The Th17 subsets are characterized by the production of IL-17, -22, and tumor necrosis factor (TNF) -α. Th17 cells provoke inflammatory responses especially in chronic conditions. Regulatory T cells (Tregs) are distinct Th subsets that suppress diverse immune responses and control the immune system balance. (Marshall et al., 2018; Bonilla and Oettgen, 2010)

1.3 Regulatory T cells

As mentioned before Tregs are part of $CD4^+$ T helper cell population. Unlike other Th cells Tregs suppress immune responses by inhibiting other immune cell (DCs, NK cells, B cells, $CD4^+$ or $CD8^+$ T cells) responses, and APCs function.

Treg cells constitute approximately 5 – 7 % of overall $CD4^+$ cells and 1 – 3 % of the peripherally circulating $CD4^+$ cells. Forkhead box protein 3 (Foxp3) is the lineage defining transcription factor of Treg cells (Fontenot et al., 2003; Attias et al., 2019). Foxp3 is essential for Treg cell development and their suppressive activity and Foxp3 dysfunctions cause autoimmune diseases (Fontenot et al., 2003). Treg cells further express T-lymphocyte associated protein 4 (CTLA-4) and CD25 surface markers and the transcription factor signal transducer and activator of transcription 5 (STAT-5) (Attias et al., 2019). CD25 is the α chain of the heterodimeric IL-2 receptor and it is constitutively expressed on Treg cells. The IL-2 signal activates STAT-5, which enhances Foxp3 expression and thus establishes the Treg cell genetic program. CTLA-4 is constitutively expressed on the surface of Treg cells (Takahashi et al., 2000) and is responsible for three characteristic Treg functions, namely TCR hyposignaling, suppression, and anergy (Tai et al., 2012). Treg cells are extremely heterogenous within the immune system and are categorized generally into two categories according to their site of differentiation: thymus-derived, natural Treg (tTreg or nTreg) cells and peripherally derived Treg (pTreg) cells (Attias et al., 2019; Sakaguchi et al., 2020). Tregs can also be induced (iTreg) *in vitro* from conventional T cells (naïve T cells) when they resemble more of pTreg cell characteristics than thymic-derived Tregs characteristic (Sakaguchi et al., 2020). The nTreg cells develop within the thymus from $CD4^+$ thymocytes with high-avidity TCR able to recognize self-antigens. The pTregs arise from conventional T cells in periphery after DC antigen activation and presence of Foxp3 inducing cytokines. The pTreg generation process is not fully known, due to lack of defined methods to distinguish pTregs from mixed Treg cell population from periphery (Lee and Lee, 2018). In periphery, tTregs are able to activate and exert suppression at very low

concentrations of peptide/MHC which ensures that upon self-antigen recognition Treg cells are the dominant responses and self-tolerance is maintained (Sakaguchi et al., 2020).

Tregs have high level of lineage stability and ability to retain their suppressive activity and Foxp3 expression in various conditions (Rubtsov et al., 2010; Sakaguchi et al., 2020). However, observations of $Foxp3^+$ cells becoming effector type cells and losing Foxp3 expression have been made which indicates their instability. Tregs can represent functional adaptability (Sakaguchi et al., 2020). They are able to gain other $CD4^+$ helper cells transcription factor and chemokine receptor profile without the inflammatory cytokine secretion. Treg cells can also adapt to the environment and contribute to maintaining the tissue homeostasis (Sakaguchi et al., 2020).

1.3.1 Transcription factor Foxp3

Foxp3 belongs to the forkhead–winged-helix family of transcription factors. It consists of a unique proline-rich N-terminal region, a central zinc finger and leucine zipper and a C-terminal forkhead (FKH) domain (Lopes et al., 2006; Lu et al., 2017). The leucine zipper domain is responsible for the Foxp3 homodimerization and the N-terminal region interacts with other partner proteins, like chromatin-modifying enzymes and transcription factors. The FKH domain mediates nuclear localization, DNA binding, interaction with nuclear factor of activated T cells (NFAT) and formation of a domain-swapped dimer.

The initiation and maintenance of Foxp3 transcription is highly dependent on few conserved non-coding sequences (CNSs), which act as binding sites for several transcription factors (Lu et al., 2017). Transcription factors binding to CNS3 initiate the Foxp3 expression whereas binding to CNS2 results in maintenance of the Foxp3 expression. The CNS1 enhancer is important for the induction of extrathymic Foxp3 expression in T cells for example in response to transforming growth factor (TGF) -β -induced SMAD signaling (Schlenner et al., 2012).

Although Foxp3 is an essential transcription factor of Tregs to maintain their phenotype and function, several studies have demonstrated that Foxp3 does not function alone (Rudra et al., 2012). Foxp3 forms protein complexes with over 300 partners (Rudra et al., 2012). Many of these partners are transcription factors such as, GATA-3, SMAD, NFAT, Runt-related transcription factor 1 (Runx1) and forkhead box protein O (FOXO) (Tone et al., 2008; Rudra et al., 2012; Ono et al., 2007; Hu et al., 2007; Ouyang et al., 2010, 2012). These factors are required to establish the distinctive transcriptional program and to define the Treg phenotype.

Mutations in *FOXP3* gene can lead to the human autoimmune immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) which is characterized by a loss of Treg function resulting in a severe autoimmunity. The syndrome can be caused by only single mutation in the *FOXP3* gene locus (Bennett et al., 2001). The IPEX patients usually develop a broad spectrum of autoimmune diseases (e.g., autoimmune nephritis, arthritis, thyroiditis, hepatitis, enteropathy, type 1 diabetes, and alopecia) and food allergies. To date, several different mutations in *FOXP3* have been identified, majority of which result in a change in the amino acid sequence in the FKH domain which is responsible of the DNA-binding of the Foxp3 protein (Le Bras and Geha, 2006; Lopes et al., 2006; Myers et al., 2006; Barzaghi et al., 2018).

1.3.2 Mechanisms of suppression

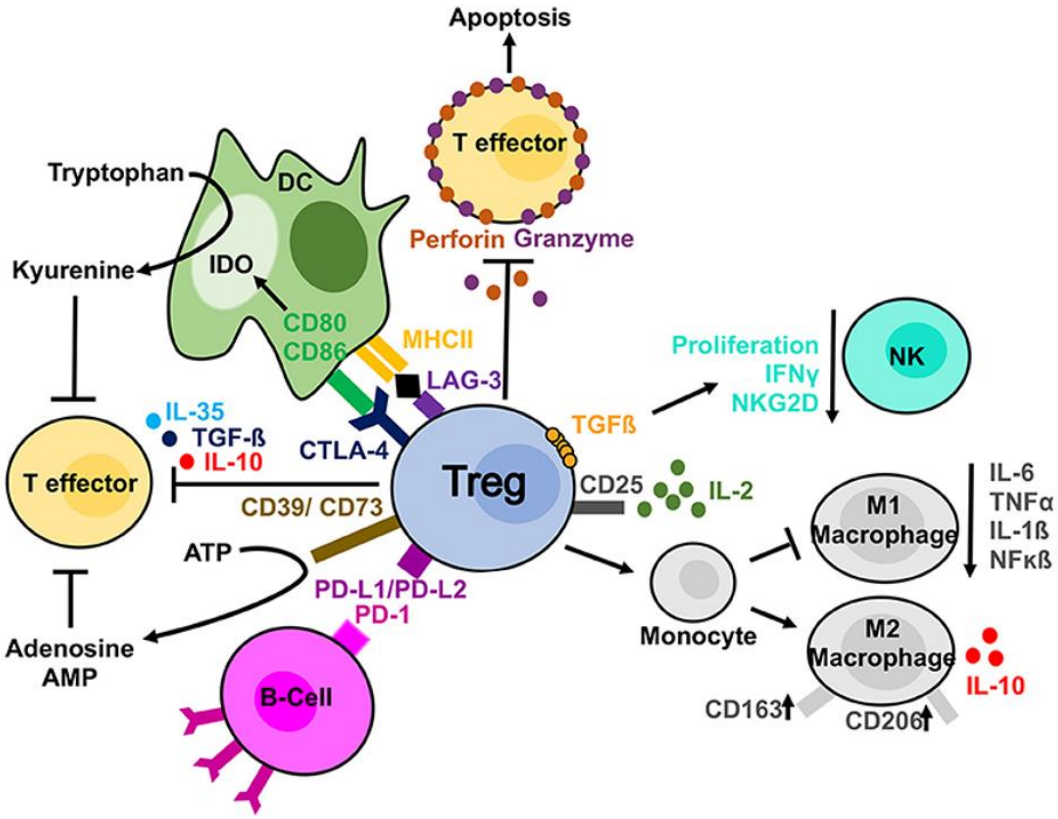


Figure 2. Regulatory T cells (Tregs) have multiple different suppressive mechanisms through which they control other cells. Tregs can produce anti-inflammatory cytokines (IL-10, IL-35, and TGF-β) which suppress other T cells. Due to high expression of CD25 they can sequester IL-2 from the microenvironment reducing proliferation of other T cells. In addition, Tregs can release perforin and granzyme, which damage target cell membrane and lead to apoptosis. Treg catalysed adenosine production through CD39 and CD73 also reduces T cell proliferation. Natural killer cells (NKs) can be directly suppressed by the membrane bound TGF-β on Tregs. Tregs have a direct effect on B-cells via PDL1/PD-1 interaction and dendritic cells (DCs) via CTLA-4 and LAG-3. CTLA-4 blocks co-stimulation and induces upregulation of indoleamine 2,3-dioxygenase (IDO). Tregs can directly act on monocytes and cause their alternative activation, which inhibits the macrophages differentiation, cytokine secretion and antigen presenting function. Figure modified from Romano et al., 2019.

Regulatory T cells can suppress variety of immune cells including $CD4^+$ and $CD8^+$ T cells, B cells, NK and natural killer T (NKT) cells, as well as DCs and monocytes (figure 2). Tregs mediate suppression via contact dependent and humoral factor-mediated mechanisms. A wide range of different cytokines (IL-2, IL-10, TGF-β, and IL-35), either directly suppress the function of conventional T cells or indirectly that of DCs (Nakamura et al., 2001; Asseman et al., 1999; Collison et al., 2007). Moreover, cell surface molecules (CD25, CTLA-4, CD39 and CD73) and intracellular or secreted molecules such as cyclic adenosine monophosphate (cAMP), granzyme and indolamine 2,3-dioxygenase (IDO) also play a major role in the suppressive function (Sakaguchi et al., 2020).

The function of these markers will be discussed in more detail in the next chapter. The main focus of this section is to discuss in more detail their role in suppressive Tregs.

One of the mechanism of Tregs to suppress APCs is through CTLA-4, which is constitutively expressed on Tregs (Takahashi et al., 2000). The CTLA-4 binds the CD80/CD86 complex and causes downmodulation of its expression on DCs together with leukocyte function-associated antigen-1 (LFA-1) (Onishi et al., 2008). Tregs have also the ability to increase expression of IDO on certain DCs. IDO catalyzes the degradation of tryptophan to kynurenine which leads to the starvation of effector T cells and to cell cycle arrest, but on the other hand it leads to the generation of Tregs (Fallarino et al., 2003; Curti et al., 2007). The increased IDO expression in DCs is also partly due to CTLA-4-induced signaling. In addition, Tregs can express lymphocyte activation gene -3 (LAG-3) which binds to MHC II with a higher affinity than its homologue CD4 (Huang et al., 2004). The binding of LAG-3 mediates the activation of different downstream pathways, which leads to the reduced capacity to capture antigens, but at the same time increased expression of co-stimulatory molecules (Huang et al., 2004).

Tregs can also suppress $CD4^+$ T cells via cytolysis, the secretion of perforin and granzymes. Tregs express granzyme A and B in a different subtypes, but both subtypes can kill autologous target cells in a perforin-dependent manner (Grossman et al., 2004b; a). The cytotoxicity has been shown to be dependent on CD18 adhesive interactions (Grossman et al., 2004a). The cytolytic suppression can be towards autologous target cells, like $CD4^+$ and $CD8^+$ T cells, and DCs in a perforin-dependent manner or B cells in a partially perforin-dependent and granzyme-B-dependent manner (Zhao et al., 2006). Tregs have also shown to suppress and kill NK and $CD8^+$ T cells in tumor microenvironment thereby suppressing the ability of immune system to clear tumors (Cao et al., 2007). In addition to perforin and granzymes, Tregs have also shown

to have other cytolytic mechanisms for suppression. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL)/death receptor 5 (DR5) pathway has been suggested to be one way Tregs can induce cell death of effector T cells (Ren et al., 2007). In addition, galectin 1, a factor able to induce T-cell apoptosis, has been shown to be upregulated on Tregs, contributing to regulatory activity *in vitro* (Garín et al., 2007).

Tregs can also trigger suppression towards B cells via the above mentioned perforin and granzyme ways (Zhao et al., 2006) and also, directly via programmed cell death 1 (PD-1) - mediated suppression (Gotot et al., 2012). Tregs can use PD-1 ligands, expressed on the cell surface, to directly suppress autoreactive B cells expressing PD-1 (Gotot et al., 2012). The B cell suppression can be also indirect through suppressing Th cells and causing the curbing of auto-antibody production (Gotot et al., 2012).

The effector T cell function can also be suppressed by extracellular adenosine, which is generated by ectoenzymes CD39 and CD73 together (Deaglio et al., 2007; Borsellino et al., 2007; Kobie et al., 2006). Adenosine activates the adenosine receptor 2A and suppresses the effector T cells. It does not only inhibit T cell function, it also promotes the generation of Tregs through promoting the production of TGF- β and inhibiting IL-6 expression (Zarek et al., 2008). In addition to adenosine, Tregs can also suppress effector T cells by transferring the inhibitory second messenger cAMP into cell via membrane gap junctions (Bopp et al., 2007).

The role of IL-2 deficiency mediated suppression, due to consumption of IL-2 via high CD25 expression is controversial. A study by Pandiyan et al. suggested that Tregs induce a cytokine (specifically IL-2)-deprivation-mediated apoptosis (Pandiyan et al., 2007). However, some studies showed that consumption of IL2 via $CD25^+$ Tregs from the microenvironment might not be the mechanism of suppression (Fontenot et al., 2005). Although Tregs require CD25 signaling under suboptimal stimulation conditions to be functionally suppressive, another study has suggested that IL-2 consumption is not a major component of Treg-mediated suppression, (Tran et al., 2009). This is in line with another study by Chinen et al. demonstrating that IL-2-deprivation by Treg cells was unnecessary for suppression of $CD4^+$ T cells, which express CD25. In contrast, they could show that IL-2 consumption by Treg cells was essential for suppression of $CD8^+$ T cell responses (Chinen et al., 2016).

Tregs have been shown to directly act on monocytes by causing the alternative activation phenotype, including the inhibition of cytokine secretion, differentiation and antigen presenting

function (Tiemessen et al., 2007). Tregs also expressed membrane-bound TGF-β, which can directly inhibit NK cell effector functions via downregulation of NKG2D receptors (Ghiringhelli et al., 2005).

1.4 Signature markers of regulatory T cells

1.4.1 Phenotype defining markers

CD25 is the alpha-chain of the interleukin 2 receptor (IL-2Rα) and constitutively expressed on Tregs at high levels. It was one of the first cell surface proteins identified on regulatory T cells (Sakaguchi et al., 1995). The CD25 and the IL-2 signaling through it is crucial for the generation, homeostasis, function and survival of the regulatory T cells (Sakaguchi et al., 1995; Fontenot et al., 2005). The expression of CD25 is not unique to Treg cells but it is rather an activation marker for all conventional T cells upon TCR activation, however the expression is significantly higher on Tregs (Workman et al., 2009).

CD127, the interleukin-7 receptor subunit alpha (IL-7Rα), is expressed on most human $CD4^+$ T cells. However, on Tregs the expression of CD127 is significantly lower than on other T cell subtypes (Hartigan-O'Connor et al., 2007). High levels of Foxp3 are detected on cell population expressing low levels of CD127 and thus an inverse correlation between CD127 and Foxp3 has been suggested (Liu et al., 2006). Cells expressing low levels of CD127 have been demonstrated to have high suppressive capacity (Hartigan-O'Connor et al., 2007; Liu et al., 2006). Some studies have also demonstrated that under certain conditions some Treg subsets express high levels of CD127 suggesting that low CD127 expression is not the only characteristic feature of Tregs (Simonetta et al., 2010). Nevertheless, for identifying pure and highly suppressive Tregs, low CD127 expression remains the general standard.

The maturation status of T cells can be determined using the CD45 cell surface marker. The different RO and RA isoforms of CD45 are expressed on T cells depending on the maturation, differentiation and activation level (Courville and Lawrence, 2021). Naïve T cells express high levels of CD45RA while CD45RO is found on primed or memory T cells (Courville and Lawrence, 2021; Prince et al., 1992). T cell activation, however, causes the loss of CD45RA and the gain of CD45RO expression. During this transition stage from naïve to memory status both isoforms of CD45 can be detected at low levels (Prince et al., 1992). The CD45 isoforms can be used to analyse the T cell population status in different conditions.

1.4.2 Co-stimulatory molecules

The CD28 is a cell surface homodimer and member of the Ig superfamily. It has an essential role for T-cell development, proliferation, survival, and cytokine production (Lenschow et al., 1996). The ligation of the CD28 receptor on Tregs and other T cells provides a critical second signal alongside the TCR ligation for naive T cell activation.

Inducible T-cell co-stimulator (ICOS, CD278) is an immune checkpoint protein that in humans is encoded by the ICOS gene. The protein encoded by this gene belongs to the CD28 and CTLA-4 cell-surface receptor family of the immunoglobulin superfamily (Hutloff et al., 1999). It forms homodimers and plays an important role in cell-cell signalling, immune responses and regulation of cell proliferation. ICOS is expressed mainly on activated $CD4^+$ T-cells following activation (Beier et al., 2000; McAdam et al., 2000). It binds to an ICOS ligand expressed by B-cells, macrophages and DCs. Its function is clearly co-stimulatory for T-cell proliferation and cytokine secretion. Loss of ICOS results in the reduction of Tregs in peripheral sites most likely due to cell-intrinsic mechanism dependent on increased sensitivity to apoptosis and loss of proliferation (Burmeister et al., 2008).

Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4, CD134) is expressed on activated $CD4^+$ and $CD8^+$ T cells but expressed constitutively on Tregs. TNFRSF4 has been identified as T-cell co-stimulatory molecule and its activation activates downstream pathways that support survival, clonal expansion and memory generation on effector T cells (So and Croft, 2007; Croft et al., 2009). The role of TNFRSF4 is more complex on Tregs than in other T cells. On Tregs the TNFRSF4 has been identified as a negative regulator of Foxp3 and the suppressive function of Tregs (Vu et al., 2007; So and Croft, 2007). TNFRSF4 co-stimulation antagonizes the conversion of naive $Foxp3^-$ CD4 T cells into $Foxp3^+$ CD4 cells (So and Croft, 2007) and induction of new inducible $Foxp3^+$ Tregs from T effector cells (Vu et al., 2007). TNFRSF4 signalling alone expands the Treg population but at the same time drives them towards exhausted state without IL-2 supplementation (Xiao et al., 2012).

Like TNFRSF4, also TNFRSF9 (CD137) is a member of the TNFR/TNF superfamily and works as a co-stimulatory molecule. The expression of TNFRSF9 is mostly induced by activation of T cells, but $Foxp3^+$ Tregs express it in a constitutive manner (Vinay and Kwon, 2011).

The co-stimulatory receptor CD27 and its ligand CD70 are members of the TNF receptor and ligand family. They have decisive roles in establishing T cell responses and memory (Hendriks et al., 2000). CD27 expressed on Treg prevents the breakdown of peripheral tolerance and limits anti-tumor immunity (Muth et al., 2021). CD27 signalling can reduce the apoptosis of Tregs *in vivo* and induces $CD4^+$ effector T cells (Teffs) to produce IL-2, a key survival factor for Tregs (Claus et al., 2012). These observations suggest that the CD27/CD70 pathway potentiates the homeostasis and inhibitory function of Tregs (Bowakim et al., 2018)

CD120b is known as TNF receptor type II (TNFR2) and it plays a pivotal role in the activation and expansion of Tregs, and some other types of immunosuppressive cells (Yang et al., 2021). The TNF signalling through CD120b has shown to be fundamental for the Tregs (Chen et al., 2007). Some evidence indicates that CD120b mediates some of the pro-Treg functions of tumor necrosis factor- alpha (TNF-α). TNF-α, for example, can induce Treg proliferation through CD120b, while CD120b signalling stabilizes Tregs phenotype and function in inflammatory environments (Chen et al., 2013). The CD120b expression on a cell can be used as a marker for Treg when it is expressed with CD25 (Chen et al., 2010). The cells expressing CD4, CD25 and TNFR2 ($CD4^+CD25^+TNFR2^+$) have been identified as Tregs more frequently than cells expressing only CD4 and CD25 ($CD4^+CD25^{high}$). Furthermore, the $CD4^+CD25^+TNFR2^+$ cell population expressed high levels of Foxp3 and other characteristic Treg phenotype markers like CCR4, CTLA-4, CD45RO, and low levels of CD127 (Chen et al., 2010).

1.4.3 Co-inhibitory molecules

PD-1 (CD279) is a co-inhibitory cell surface receptor which belongs to the CD28 superfamily. PD-1 is expressed on activated T cells and other lymphocytes, and the expression level increases with prolonged antigen exposure and stimulation. PD-1 is highly expressed on activated Tregs, but its function remains unclear. Tregs expressing high levels of PD-1 have reduced *FOXP3* gene demethylation, weaker suppression towards conventional T cells, low amount of proliferation *in vitro* and relatively short telomerases, which all support the idea of terminal cell differentiation and exhaustion status (Lowther et al., 2016). A mice study by Wong et al. has shown that blockade of PD-1 with antibody induced the formation of adaptive regulatory T cells and that Tregs expressing low PD-1 levels had a high capacity to promote B cell apoptosis and suppress helper T cells (Wong et al., 2013). Moreover, PD-1 expression on Tregs has a role in maintaining the peripheral tolerance. On the other hand, when lowly

expressed it has been linked to onset of autoimmune conditions and when highly expressed to tumor progression (Francisco et al., 2010; Gianchecchi et al., 2013; Zhulai and Oleinik, 2022).

The CTLA-4 (CD152) is a co-inhibitory surface receptor, known for its suppressive function, and a close relative of CD28 sharing the same ligands (CD80, CD86). CTLA-4 expressed by activated conventional T cells is constitutively expressed by Tregs (Takahashi et al., 2000). CTLA-4 has a key role in maintaining the immune homeostasis and the heterozygous loss of it causes severe autoimmunity (Schubert et al., 2014). CTLA-4 has cell-intrinsic and cell-extrinsic functions, through which it contributes to immunological tolerance and immune responses (Wing et al., 2011). One of the most important cell-extrinsic functions of CTLA-4 is to control the availability of CD80 an CD86, two co-stimulatory molecules on antigen presenting cells which are needed for cell activation. CTLA-4 deficiency causes impairment of suppressive function of Tregs by affecting the ability to down-regulate CD80 and CD86 expression on antigen presenting cells (Wing et al., 2008). This implicates that CTLA-4 expression on Tregs is crucial to maintain suppressive immune responses.

Lymphocyte activation gene -3 (LAG-3, CD223) is a transmembrane molecule having a co-inhibitory function. The conformation of LAG-3 resembles the CD4 co-receptor and it also binds to the MHC II like CD4, but with a greater affinity (Huard et al., 1995). LAG-3 is recognized as an important immune checkpoint molecule that controls T cell activation. LAG-3 knock-out model mice had higher levels of $Foxp3^+$ Tregs, but the functional profile of them was defective (Lee and Min, 2020). The same study introduced the important role of LAG-3 intracellular signalling to Treg suppressive function. A study conducted by Huang et al. found that natural Tregs express LAG-3 upon activation and the expression is significantly enhanced in the presence of effector cells, whereas Tregs derived from LAG-3 knock-out mice exhibit reduced regulatory activity (Huang et al., 2004). The study also discovered that ectopic expression of LAG-3 on $CD4^+$ T cells confers suppressor activity towards effector T cells, which indicates that LAG-3 might be a marker of the Treg population and contributes to the suppressive activity.

T cell immunoreceptor with Ig and ITIM domains (TIGIT) is a co-inhibitory molecule that belongs to the immunoglobulin superfamily and is expressed only on lymphocytes including regulatory, memory and activated T cells (Yu et al., 2009). TIGIT has been reported to inhibit immune cell responses through multiple ways like impairing T cell priming by DCs, preventing tumor cell killing by NK cells and cytotoxic T cells, and enhancing the immune suppressive

activity of regulatory T cells (Harjunpää and Guillerey, 2020). The expression of TIGIT on Tregs divides the regulatory T cells into different subsets (Joller et al., 2014). TIGIT$^+$ Treg cells showed high ability to suppress TCR-stimulated proliferation of T cells and thus TIGIT marks Tregs with superior suppressive capacity. The phenotype and transcriptional profile of TIGIT$^+$ Tregs suggested a highly activated and suppressive subtype with high signature gene expression when compared to Tregs lacking TIGIT (Joller et al., 2014).

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3, CD366) is also a co-inhibitory molecule. The TIM-3 is mostly expressed on Th1 and CD8$^+$ T cytotoxic 1 T cells, but it has also been detected on Tregs and on innate immune cells (Anderson et al., 2016). The expression of TIM-3 is a marker of highly suppressive tissue-resident regulatory T cells that has an important role in the antitumor immune responses (Sakuishi et al., 2013). Sakuishi et al could show that the majority of the intratumoral Foxp3$^+$ Tregs express TIM-3 (Sakuishi et al., 2013). Moreover, another study revealed that the majority of tumor-derived TIM-3$^+$ CD4$^+$ T cells express also high levels of CD25, Foxp3, CTLA-4 and glucocorticoid-induced TNFR-related protein (GITR) (Yan et al., 2013), which are characteristic markers for regulatory T cells. These findings indicate that Foxp3$^+$ CD4$^+$ T cells expressing TIM-3 in human tumors represent functional regulatory T cells.

1.4.4 Other markers

MHC class II cell surface receptor encoded by the human leukocyte antigen (HLA-DR) is typically expressed on antigen-presenting cells. Interestingly HLA-DR is also found on T cells and has been associated with T cell activation. CD4$^+$CD25$^+$ regulatory T cells expressing HLA-DR are a distinct population of Tregs, which are highly and rapidly suppressive (Baecher-Allan et al., 2006). These cells suppress T cells through an early contact dependent mechanism that is associated with Foxp3 induction.

C-C chemokine receptor type 4 (CCR4) also known as CD194 is mostly expressed by the Th2 cells, and to a lesser extent by Tregs (Yoshie and Matsushima, 2015). CCR4 mediates the migration of Tregs to its ligands, CCL17 and CCL22, which are produced by DCs upon maturation, thereby playing a key role in recruiting Tregs into lymphoid tissues (Gobert et al., 2009; Maeda et al., 2019). CCR4 can also be used as marker of differentiated and most suppressive CD45RA$^-$Foxp3^{hi}CD4$^+$ Treg cell subset, because it is specifically expressed by them, not by naïve Tregs (Sugiyama et al., 2013). In cancer patients, CCR4$^+$ Tregs are the most

prominent Treg subtype in tumors in much higher amounts than in peripheral blood (Sugiyama et al., 2013; Gao et al., 2022).

CCR7 (CD197) is another C-C motif chemokine receptor, which mediates the migration of naive T cells into the secondary lymphoid organs and stimulates DC maturation (Schneider et al., 2007). Moreover, CCR7 controls the recruitment of lymphocytes and mature DCs to lymph nodes and other lymphoid tissues (Saban, 2014). CCR7 is also needed for the negative selection of auto-reactive lymphocytes in the thymic medulla (Hu et al., 2017; Kurobe et al., 2006). A CCR7 knock-out mouse study has shown that CCR7 is required for the Treg migration into the lymph nodes and also to their suppressive function *in vivo* (Schneider et al., 2007). Additionally, CCR7 limits the thymus recirculation of Tregs, but it is not affecting their production or emigration (Cowan et al., 2016).

Ecto-5′-nucleotidase (CD73) and ectonucleoside triphosphate diphosphohydrolase-1 (CD39) are ectonucleotidases which jointly catalyze the extracellular adenosine generation from nucleotides like adenosine triphosphate (ATP) and adenosine monophosphate (AMP) through hydrolysis (Deaglio et al., 2007). The generated adenosine has been shown to inhibit effector T cell function through binding to its receptor and activating cell intrinsic pathways, which suggests that adenosine participates in the suppressive function of Tregs (Smyth et al., 2013; Deaglio et al., 2007). Both, CD39 and CD73, are expressed by Tregs and identified as functional Treg surface markers (Mandapathil et al., 2009; Deaglio et al., 2007). As the level of adenosine affects the microenvironment and has suppressive functions, the frequency of the CD73/CD39 expressing cells varies in disease conditions. Studies have observed an increased number of circulating $CD39^{+}$ Tregs in cancer patients and decreased levels in some autoimmune diseases (Timperi and Barnaba, 2021; Borsellino et al., 2007).

C-X-C motif chemokine receptor 3 (CXCR3, CD183) is a chemokine receptor that plays an important role in T cell trafficking and function (Groom and Luster, 2011). CXCR3 with its ligands is an inflammatory chemokine system that is capable of coordinating T cell responses in the inflamed periphery. It has also a role in the generation of both suppressive and inflammatory T cell responses. In context of Tregs, CXCR3 has been reported to recruit Tregs to the site of inflammatory reaction (Oo et al., 2010) and transplant rejection (Hasegawa et al., 2007). The expression of CXCR3 may also indicate that Treg has adopted the Th1-like profile. Induction of naïve Tregs with IL-12 resulted in upregulated expression of Th1-associated

transcription factor T-box expressed in T cells (T-bet), IFN-γ, and CXCR3 (McClymont et al., 2011).

CXCR4 (CD184) is another C-X-C motif chemokine receptor with important roles in hematopoiesis, development, and organization of the immune system. The ligand of CXCR4, CXCL12, functions as an anti-inflammatory chemokine in inflammatory responses and also participates in regulating of immune surveillance, cancer, and autoimmunity (Karin, 2010). The CXCL12 is constitutively expressed in several tissues, for example in bone marrow, which enables it to regulate the localization and trafficking of leukocytes and other cells (Karin, 2010). The CXCL12 has been shown to mediate the effector T cell polarization towards antigen-specific Tregs through the CXCR4 (Meiron et al., 2008). Overexpression of CXCR4 on Tregs has been shown in multiple cancers, like breast cancer and renal cancer (Yan et al., 2011; Santagata et al., 2017). A study by Yan et al. reported that high Treg infiltration in the tumor environment correlates with CXCL12 expression and the up-regulation of CXCR4 expression on Tregs due to hypoxia (Yan et al., 2011).

CD15s (sialyl Lewis x) is a sialylated form of CD15 and highly expressed by monocytes, granulocytes and activated lymphocytes, especially T cells. Miyara et al. showed that CD15s is expressed by activated, most suppressive, and terminally differentiated Foxp3^{high} Treg cells and thus can be used as marker between non-suppressive, inflammatory cytokine secreting T cells and suppressive Tregs (Miyara et al., 2015). However, the CD15s does not seem to be significant for the suppressive function of Tregs, because its blocking with anti-CD15s mAb did not have an effect to the suppressive activity of Tregs *in vitro* (Miyara et al., 2015). Overexpression of CD15s has been also linked to higher tumor metastasis, recurrence of cancer, and lower overall survival of the cancer patients (Liang et al., 2016).

CD38 is also known as cyclic ADP ribose hydrolase and it has a role in several processes, including cell migration, adhesion, and signal transduction. CD38 is expressed widely by immune cells, especially T cells, and was first identified as an T cell activation marker (Morandi et al., 2019). CD38 has been found to work also in the adenosine production, but from a different axis (CD38/CD203a/CD73) than previously known CD39/CD73 pathway (Horenstein et al., 2013). In this axis the adenosinergic loop is initiated by CD38 converting NAD^+ to cyclic ADP ribose (cADPR), which is then metabolised by CD203a to AMP, which in turn is converted to adenosine by CD73. The importance of CD38 on Treg function has also been noticed via knockout models. Tregs expressing CD38 at high level had superior suppressive activity

compared to Tregs with low CD38 expression (Patton et al., 2011). In the same study, Tregs expressing low levels of CD38 failed to upregulate the CD73 expression, indicating the important role of CD38 for Treg suppressive function (Patton et al., 2011).

CD103 is an alpha E integrin (ITGAE) that mediates cell migration, adhesion, and lymphocyte homing of cell through interaction with E-cadherin, a protein expressed on epithelial cells (Kim et al., 2019). The CD103 is expressed on T lymphocytes, but also on DCs and mast cells. The expression of CD103 on $CD4^+$ T cells has been linked to the regulatory phenotype marker (Allakhverdi et al., 2006). $CD103^+$ cells showed a high suppressive capacity and an increase in Foxp3 mRNA, regardless of CD25 coexpression (Allakhverdi et al., 2006). This suggests that $CD4^+$ T cells expressing CD103 are Tregs. The expression of CD103 has also been found to be upregulated on the tumour infiltrating Tregs, especially in tumours secreting TGF-β (Anz et al., 2011). TGF-β is known to promote CD103 protein expression through enhancing its gene transcription (Allakhverdi et al., 2006; Robinson et al., 2001). The suppressive capacity of tumor infiltrating $CD103^+$ Tregs has shown to be higher towards effector T cells than CD103 negative Tregs (Anz et al., 2011).

1.5 Health and disease

Treg cells are important in maintaining the normal homeostasis and suppressing unwanted or too strong immune reactions. However, abnormalities in Treg numbers, frequencies, or suppressive function can trigger autoimmune diseases (Attias et al., 2019; Sakaguchi et al., 2020). Some key mechanisms that can lead to Treg cell dysfunction in human diseases are: genetic defects, presence of Treg cell destabilizing factors, abrogation of Treg cell-promoting signals and co-opting Treg cell suppressive function (Attias et al., 2019).

One of the most severe examples of Treg cell dysregulation is the IPEX syndrome described in detail under 1.3.1 section. Treg cells are engaged in supressing immune reactions against self-constituents, environmental subsets, and microbes, and that is why the dysfunction or deficiency of them can cause autoimmune diseases and allergies in humans (Sakaguchi et al., 2020). Therefore, disruptions in Treg cell signature genes, receptors or pathways can lead to autoimmune disease due to dysfunctional Treg cells. In case of deficient Tregs, self-reactive T cells can be activated and expanded without control causing autoimmune reactions (Sakaguchi et al., 2020).

Other gene defects can also cause Treg abnormalities and dysfunction. A rare autosomal recessive deficiency in CD25 has been detected and it has been recorded to manifest like IPEX syndrome (Sharfe et al., 1997; Caudy et al., 2007; Goudy et al., 2013; Bezrodnik et al., 2014). IL-2 is essential for survival and function of Tregs and also for the initiation and maintenance of adaptive T-cell responses. The disease manifestation is various, but often it includes different infections, enteropathy, lymphadenopathy, hepatosplenomegaly, eczema, and less often diabetes, autoimmune thyroiditis and asthma (Cepika et al., 2018). In addition, CTLA-4 deficiency can lead to uncontrolled effector T cell responses due to abnormal Treg function. Animal studies have shown that loss of CTLA-4 in mice results in lethal systemic autoimmunity (Tivol et al., 1995). In humans, CTLA-4 haploinsufficiency leads to immune dysregulation (Kuehn et al., 2014). The disease is known as CTLA-4 haploinsufficiency with autoimmune infiltration (CHAI) disease (Lo et al., 2016) and presents with autoantibody-mediated cytopenia, hypogammaglobulinemia, lymphadenopathy, organ specific autoimmunity, lymphoproliferation, lymphocytic infiltration of nonlymphoid organs and neurological involvement (Lo et al., 2016). Deficiency of CTLA-4 can also lead to LPS responsive beige-like anchor protein (LRBA) deficiency, characterized by autoantibodies, Treg cell defects, autoimmune infiltration and enteropathy (LATAI) disease. LATAI disease shares clinical and phenotypical features with CHAI (Lo et al., 2016; Gámez-Díaz et al., 2016). There are also other known mutations, e.g., mutations in *BACH2* region, itchy E3 ubiquitin protein ligase (ITCH), IL-2 inducible T cell kinase (ITK) or *STAT-3*, that can cause the downstream dysregulation of Tregs (Cepika et al., 2018).

In cancer, Tregs can suppress antitumor responses, worsening disease outcome (Attias et al., 2019; Sakaguchi et al., 2020). The Treg cell suppression function in tumour immunity can hamper the immune system ability to destroy the tumour cells. This increased Treg function and immune suppression contributes to cancer onset, progressions and ultimately to metastases. In the tumour microenvironment, Tregs are in a highly proliferative state expressing high levels of T cell activation associated molecules (e.g., CD25, CTLA-4, ICOS, OX40, LAG-3, GITR, TIGIT, and PD-1). In addition, tumour cells contribute to Treg cell generation by expressing IDO, which also leads to the starvation of effector T cell (Attias et al., 2019). In addition, within the tumour microenvironment Treg cells mediate immunosuppression and thereby supress and prevent other immune cells from destroying the tumour cells. High frequencies of tumour infiltrating Tregs compared to $CD8^+$ T cell frequencies are correlating with poor prognosis and high metastatic potential (Attias et al., 2019; Sakaguchi et al., 2020).

1.6 Regulatory T cell modulation as therapeutic option

In autoimmune diseases Treg cells are not working properly, or they are absent. This causes an inadequate immune response, and uncontrolled inflammatory response of effector T cells, which has raised interest towards Treg cells as immunotherapeutic target. There are two different approaches to treat autoimmune diseases: cell-based therapies and non-cell-based therapies, both aiming to restore and enhance Treg cell balance and function *in vivo* (Eggenhuizen et al., 2020).

The non-cell-based therapies include for example low-dose IL-2, TNFR2 agonists, rapamycin and autoantigens. IL-2 can activate Treg cells and has potential to expand Treg cell in patients *in vivo* (Abbas et al., 2018). When low dose of IL-2 is used, only $CD25^{high}$ Treg cells are targeted (Tang, 2015) promoting Treg cell differentiation, expansion, and action. TNFR2 is a receptor for TNF-α, which is able to induce anti-inflammatory and tissue regenerative effects. Treg cells have high expression of TNFR2 and its expression is linked to Treg suppressive capability (Chen et al., 2013, 2010). Nevertheless, there is yet no TNFR2 agonist in clinical use. Rapamycin is a mTOR inhibitor and it is very potent macrolide immunosuppressant drug (Thomson et al., 2009). It inhibits DCs ability to mature and stimulate T cell differentiation and activation. On the other hand, rapamycin selectively expands Treg cells and helps maintaining their function and suppressive phenotype. This results in enrichment and induction of Tregs promoting immune tolerance. Autoantigens have also been suggested as therapeutic option to activate and expand specific Treg cells. Different DNA vaccines, proteins, peptides, and epitopes loaded to carriers have been used in clinical trials and found to be safe and well tolerated (Eggenhuizen et al., 2020).

The cell-based therapies include approaches of using polyclonal Treg cells, autoantigen-specific TCR and CAR (chimeric antigen receptor) Treg cells, and also, tolerogenic DCs (Eggenhuizen et al., 2020). The polyclonal Treg cells offer a non-specific way to restore Treg cell balance with the use of these *ex-vivo*-expanded cells with natural repertoire of TCRs. When generating antigen-specific TCRs or CARs onto Tregs, the Treg can be directed to specifically target and activate the auto-antigenic immune responses (Selck and Dominguez-Villar, 2021; Raffin et al., 2019). At the moment, several clinical trials using different Treg cell-based therapies are ongoing to treat autoimmune diseases (Eggenhuizen et al., 2020; Selck and Dominguez-Villar, 2021).

In cancer, Treg cells are overactive and supress anti-tumour immunity allowing the tumour to progress and ultimately metastasize. This makes Treg cells also a potential target for cancer immunotherapies through their depletion or controlling their function. Specifically, markers important for Treg cell function, such as CD25, CTLA-4, ICOS and PD-1 are targeted. Also, chemokine receptors like CCR4 and CCR8 can be targeted to deplete Treg cells. The use of immune-checkpoint inhibitors (ICIs) has recently increased rabidly as treatment option for different cancers (Togashi et al., 2019). While ICIs targeting PD-1 signalling pathways are part of the standard therapy against several malignancies, not all patients benefit from anti PD-1 or anti PDL-1 treatment. However, in some cases, blocking PD-1 signalling has shown to enhance the immunosuppressive function of Treg cells, whereas CTLA-4 inhibitors have caused Treg cell death (Togashi et al., 2019). Another approach to prevent the harmful effect of Treg cells in cancer is to modulate the function of the cells and inhibit the cell infiltration to the tumour site. This can be done by targeting metabolic pathways, cell signalling, and chemokine and cytokine secretion (Ohue and Nishikawa, 2019).

Although, there has been a huge advance in therapy options utilizing Tregs, further studies are needed to study the long-term effect and solve the challenges of each treatment (Selck and Dominguez-Villar, 2021; Raffin et al., 2019; Goswami et al., 2022). In addition, understanding the mechanisms in prevention of cancer, autoimmunity and other diseases in humans is essential (Chen et al., 2022; Eggenhuizen et al., 2020).

1.7 Aim of the study

The overall aim of this study was to characterize human iTregs at single cell level using mass cytometry. For this purpose, a human iTreg antibody panel will be set up and validated. This antibody panel will be then applied to profile early differentiation of human iTreg cells.

2 Results

2.1 Human iTregs expressed Foxp3 and were suppressive

For each iTreg cell cultures, $CD4^+$ cells, isolated from single cord blood donor were activated and differentiated into Th0 and iTreg cells. Naïve $CD4^+$ T cells were characterized by the expression of memory-associated surface marker CD45RO and naïve-associated surface marker CD45RA using flow cytometry. Cells with naivety greater than 60% were used for cell differentiation.

The successful iTreg cell differentiation was verified at 72 h by measuring the expression of Foxp3 at protein level using western blot analyses and intracellular flow cytometry staining.

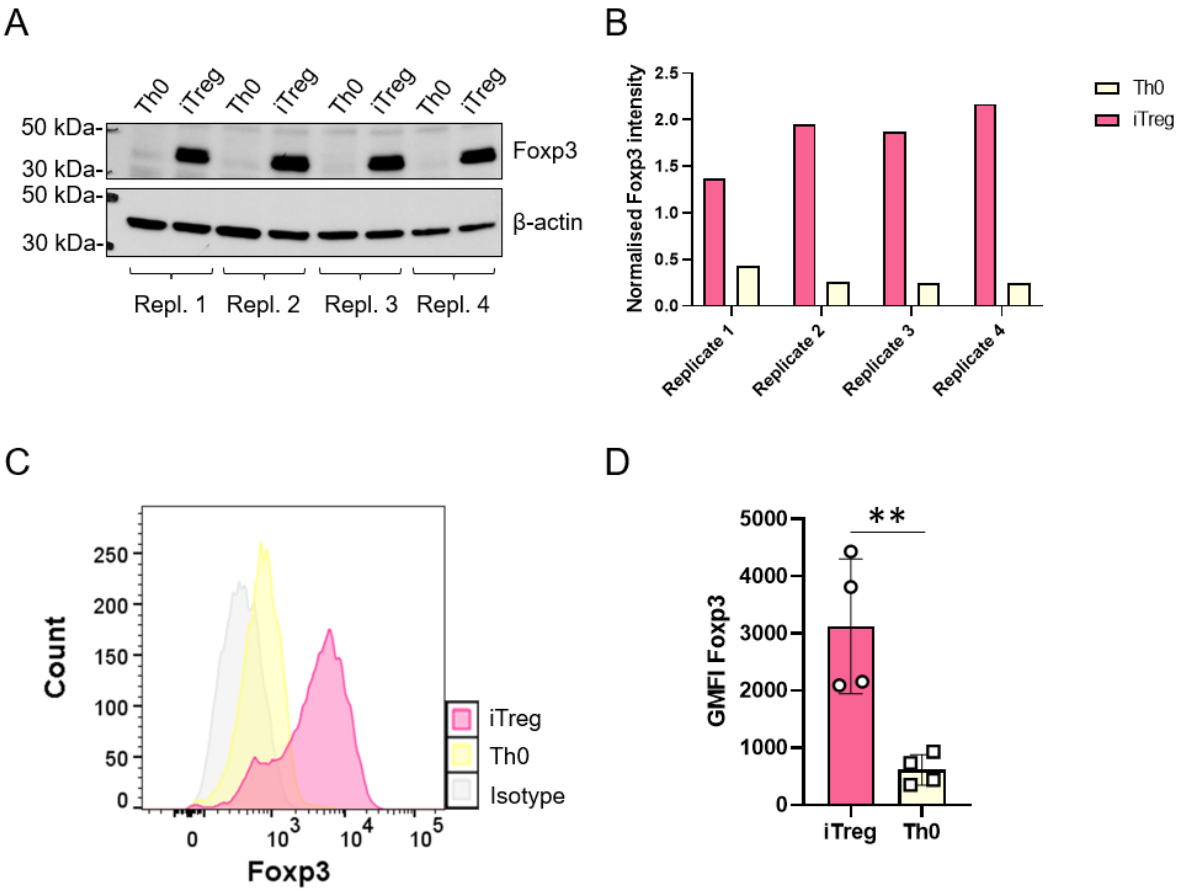


Figure 3. Foxp3 was upregulated in iTregs at 72h. A) At 72h of differentiation, immunoblotting showed a strong upregulation of Foxp3 in iTregs compared to activated control cells (Th0) in four individual donors (Repl. 1-4). B) The protein band intensities, normalised to β-actin, support the increase of Foxp3 expression. C & D) The Foxp3 expression was also confirmed by flow cytometry, showing a significant upregulation of the mean fluorescence intensity (GMFI) of Foxp3 in iTregs from one representative donor (C) and four individual replicates (D). The GMFI data is represented as mean ± standard deviation (SD) for four individual replicates. (**p<0.01).

The expression of Foxp3 at protein level was strongly upregulated in iTregs from each single donor at 72 h (figure 3A). The signal intensities of the protein bands supported the increased expression of Foxp3 in iTregs (figure 3B). Furthermore, the flow cytometry analyses confirmed the upregulation of Foxp3 to be statistically significant ($p < 0.05$) at 72 h in iTreg cells compared to Th0 cells (figures 3C & D).

The suppressive capacity is one of the main characteristics of Tregs. The suppressive capacity of iTregs was assessed by flow cytometry after a four-day co-culture with fluorescence-labeled responder cells at different ratios (1:1, 1:0.5 and 1:0.25). The amount of suppression was determined by quantifying the percentage of proliferating responder cells. High percentage of proliferating cells indicates weak suppressive capacity.

iTreg cells suppressed the responder cell proliferation at all ratios compared with Th0 control cells. The strongest effect was observed at ratios (responder cells : iTreg) 1: 0.5 ($p = 0.07$) and 1:0.25, showing significantly higher iTreg suppression compared to Th0 suppression (figures 4A & B).

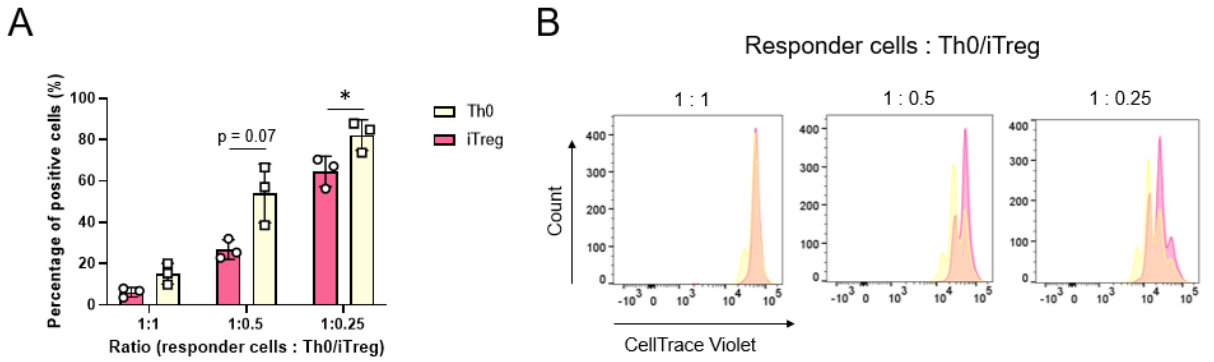


Figure 4. The suppressive capacity was induced in iTregs. To assess the suppressive capacity of iTregs and Th0, cells were cocultured at different ratios with proliferating $CD4^+$ cells. The percentage of proliferating fluorescent labeled CD4+ cells (responder cells) was measured by flow cytometer. Lower percentages indicated stronger suppression of proliferating CD4+ cells. A) iTregs had statistically significant stronger suppressive capacity compared to Th0 at ratio 1:0.25. B) One representative replicate showing the proliferation of CD4+ cells cocultured with Th0 or iTreg. Lower intensities of CellTrace Violet reveal divided cells. The data in (A) is represented as mean ± SD for three individual replicates. (*$p<0.05$).

2.2 Characteristic phenotype markers were present on iTregs

The Treg phenotype has been reported to be $CD127^{low}$ $CD25^{high}$ $Foxp3^{high}$ (Hartigan-O'Connor et al., 2007; Liu et al., 2006). The expression of Foxp3 increased in iTregs during differentiation and with significantly higher (≥ 30%) $Foxp3^{+}$ cells in iTregs compared to the Th0 controls at all given time points (figure 5A, left panel). In addition, the mean fluorescence intensity (MFI) of Foxp3 was also statistically significantly higher upon Treg cell differentiation compared to Th0 cells (figure 5A, right panel).

On the other hand, the expression level of CD25 was high on both iTregs and Th0 cells at all measured time points and no statistically significant differences were detected between them (figure 5B, left panel). However, the MFI values of CD25 were significantly higher on iTregs than on Th0 cells at 24h (figure 5B, right panel). At 72h, a slight upregulation of CD25 on iTregs was observed, but the difference between iTregs and Th0 cells was not statistically significant (MFI: $p = 0.08$, expression %: $p = 0.10$).

The percentage of CD127 positive cells was low on both iTregs and Th0 cells over time (≤ 10%, figure 5C), and no statistically significant difference was observed (24h: $p = 0.06$, 48h: $p = 0.59$, 72h: $p = 0.22$).

During cell activation, Th0 and iTreg cells gained the memory cell marker CD45RO (figure 5D). At 72h, the percentage of CD45RO positive cells was statistically significantly higher on iTregs compared to Th0 cells (figure 5D, left panel). The MFI values confirmed the same trend as described in CD45RO positive iTregs cells at 72h (figure 5D, right panel). In addition, at 24h timepoint the MFI values were statistically significantly higher on iTregs than on Th0 cells.

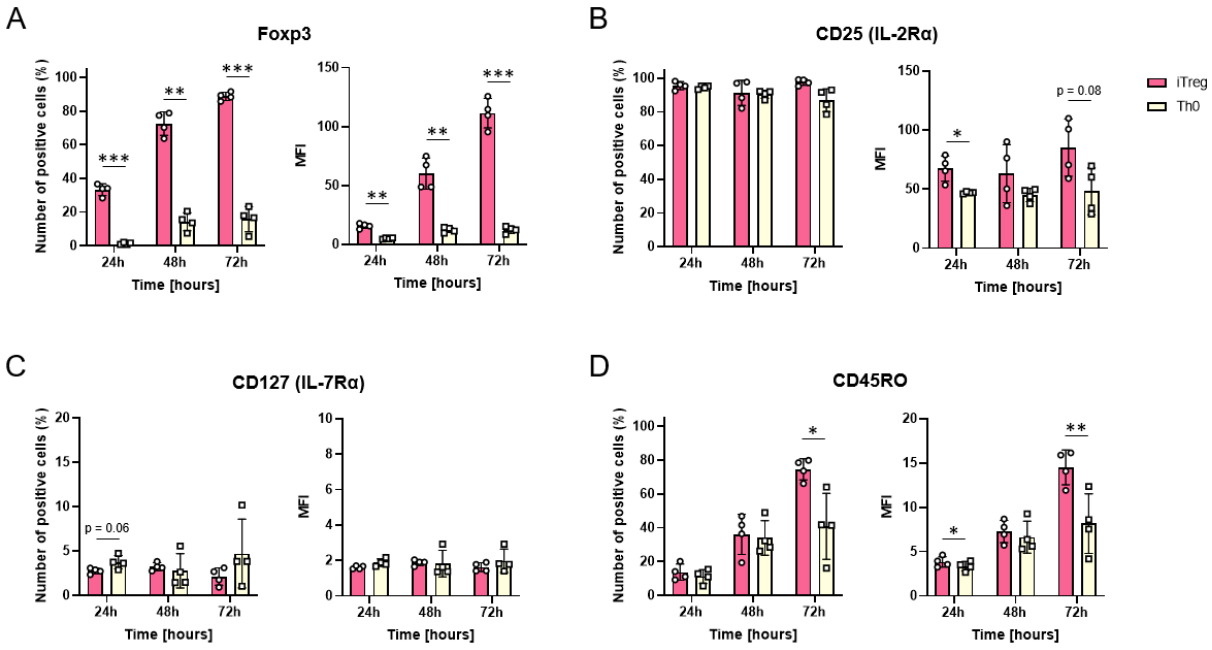


Figure 5. The characteristic phenotype markers were expressed on iTregs. Naïve CD4⁺ cells were isolated from 4 individuals and differentiated into iTreg cells. Activated Th0 served as control. Cells were stained at 24h, 48h and 72h and applied to mass cytometry. Percentage of positive cells and mean fluorescence intensity (MFI) of each marker A) Foxp3, B) CD25, C) CD127, and D) CD45RO are shown. The data are represented as mean ± SD for four individual replicates. (*p<0.05, **p<0.01, ***p<0.01).

2.3 Expression of co-inhibitory markers on iTregs increased during cell differentiation

The inhibitory molecules are crucial for the Treg function. Several of the inhibitory molecules measured in this study were upregulated with time. PD-1 was exceptionally highly expressed on iTregs at all timepoints (figure 6A). The MFI values of PD-1 increased with time both on iTregs as well as Th0 cells, though the expression was statistically significantly higher in iTregs at all timepoints (figure 6A, right panel). The percentage of PD-1 positive iTregs was nearly 100% at early and late differentiation stages (figure 6A, left panel). Nevertheless, at early differentiation stage (24h), the expression of PD-1 was statistically significantly higher on iTreg than on Th0 cells (figure 6A, left panel).

The expression of CTLA-4 increased with time in Th0 and iTreg cells, nevertheless, no statistically significant difference was observed between iTregs and Th0 cells (figure 6B).

The percentage of TIM-3 and TIGIT positive iTreg cells was relatively low at early time points (< 1 %) (figures 6C & D). The expression of TIM-3 increased with time and was statistically significantly higher on iTregs compared to Th0 cells at 48 and 72h. TIGIT, on the other hand,

was not statistically significantly upregulated on iTregs, although a slight trend towards higher expression on iTregs was observed (figure 6D).

LAG-3 expression was also upregulated on iTregs compared to Th0, but the upregulation was not statistically significant (24h: p = 0.06, 48h: p = 0.12, 72h: p = 0.55) (figure 6E).

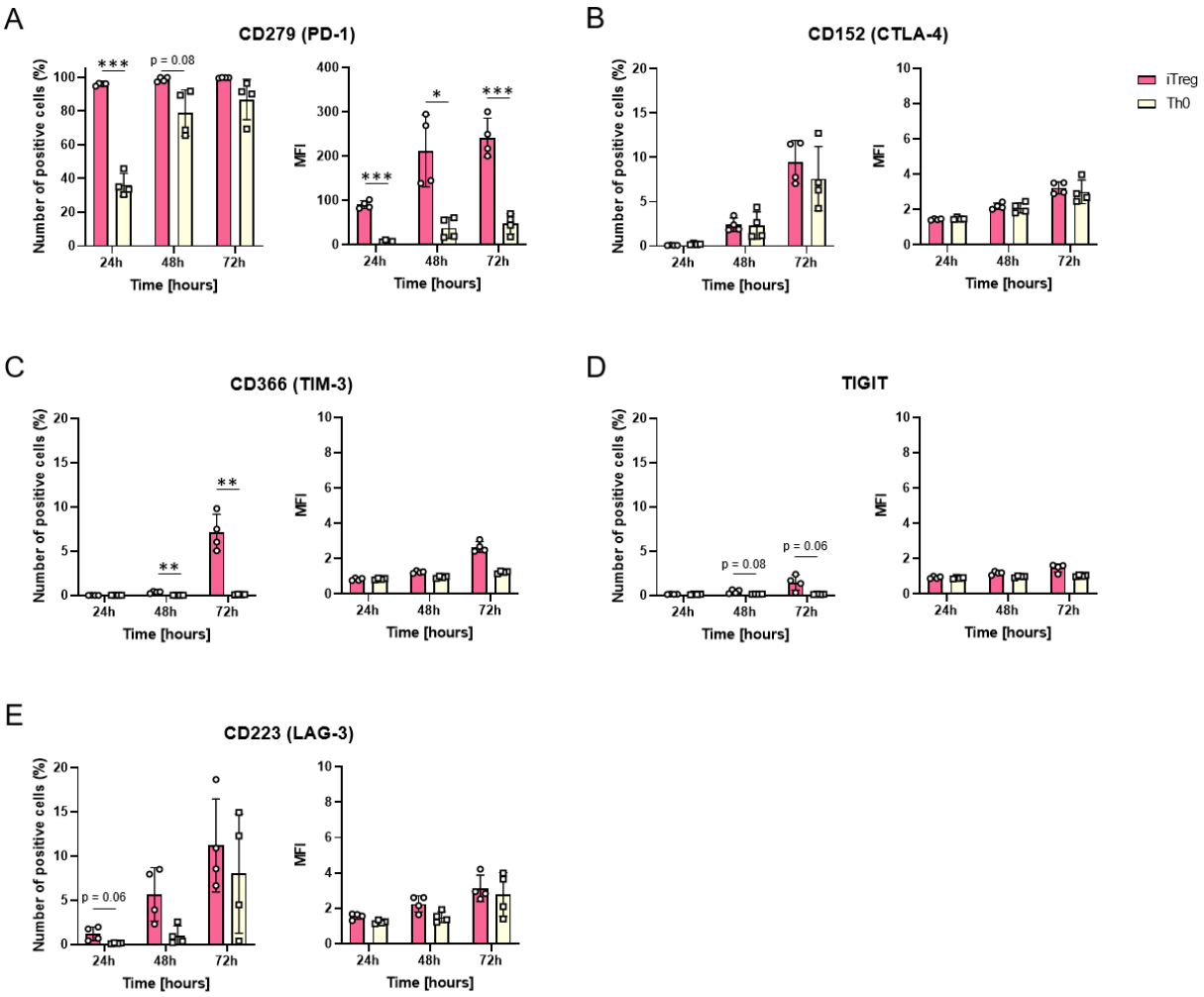


Figure 6. Expression of inhibitory markers increased during cell differentiation. Naïve CD4⁺ cells were isolated from four individuals and differentiated into iTreg cells. Activated Th0 cells served as control. Cells were stained at 24h, 48h and 72h and applied to mass cytometry. Percentage of positive cells and mean fluorescence intensity (MFI) of each inhibitory marker A) CD279, B) CD152, C) CD366, D) TIGIT, and E) CD223 are shown on iTregs and Th0 controls. The data are represented as mean ± SD for four individual replicates. (*p<0.05, **p<0.01, ***p<0.01).

2.4 CD103 and several chemokine receptors were upregulated on iTregs

CD103, also known as integrin αEβ7 (ITGAE) was expressed at a high level on iTregs whereas no expression was observed on Th0 cells (expression level < 0.1 %), suggesting that CD103 is a characteristic iTreg marker in our experimental setting (figure 7A). The percentage of CD103 positive cells was significantly upregulated on iTregs at early and late time points (figure 7A, left panel). Up to 20% and 60% of CD103 positive iTregs were detected at 48 and 72h, respectively. MFI values confirmed its significantly higher expression on iTregs at 48 and 72h compared to Th0 (figure 7A, right panel).

Both C-C motif chemokine receptors, 4 and 7, were strongly upregulated on iTregs compared to Th0 cells. The percentage of CCR4 (CD194) positive iTregs was significantly higher at 24, 48 and 72h compared to Th0 cells (figure 7B, left panel), which was further confirmed by the MFI data (figure 7B, right panel). However, donor dependent variation in CCR4 expression was observed in both iTregs and Th0 cells, as seen from the high standard deviations. The percentage of CCR7 (CD197) positive cells and its MFI were significantly upregulated on iTregs at all timepoints, with highest expression at 24h, when compared to Th0 cells (figure 7C). Interestingly, Th0 cells showed a decrease in CCR7 expression with time, which was not observed in iTregs. However, the expression of CCR7 also varied between the four individuals.

The percentage of C-X-C motif chemokine receptor 3 (CXCR3, CD183) positive iTregs cells (60% at early and 90% at late time points) was significantly higher ($p < 0.01$) at all timepoints when compared to Th0 (figure 7D, left panel). The MFI values followed almost the same trend showing a statistically significant upregulation of CXCR3 on iTregs at 24 and 72h timepoint, but not at 48h timepoint ($p = 0.051$) compared to Th0 cell controls (figure 7D, right panel).

The CXCR4 (CD184) was expressed on both cell types, but no significant difference could be detected between iTreg and Th0 cells (figure 7E).

CD38 was very highly expressed on both iTregs and Th0 cells at all timepoints (figure 7F, left panel). A statistically significant upregulation of CD38 positive cells was detected on iTregs at the 72h compared to Th0 positive cells. MFI values of CD38 did not show any statistically significant difference at any timepoint, although the MFI was slightly higher on iTregs at 72h ($p = 0.07$) (figure 7F, right panel).

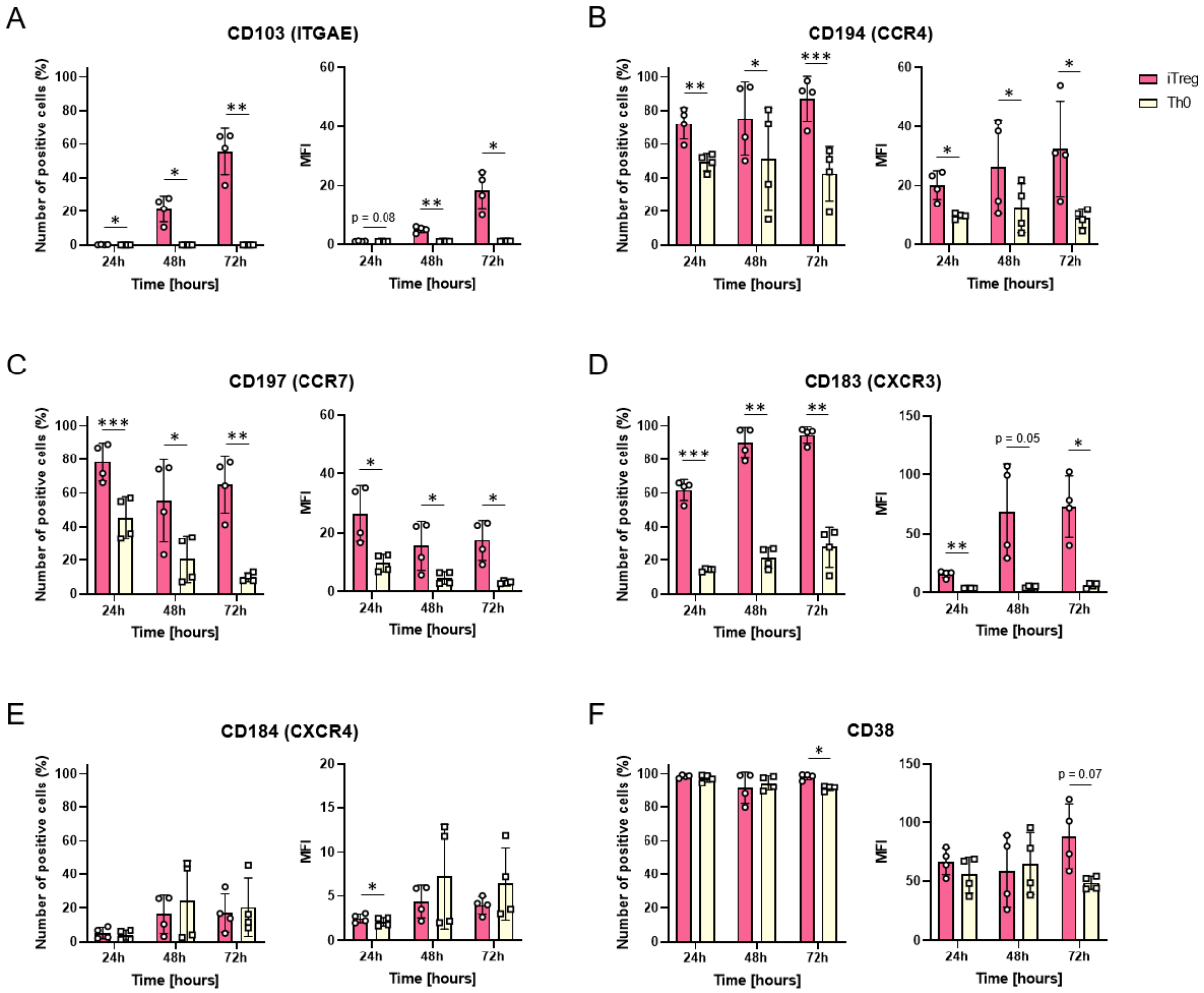


Figure 7. CD103 and several chemokine receptors were upregulated on iTregs compared to Th0 cells. Naïve CD4⁺ cells were isolated from 4 individuals and differentiated into iTreg cells. Activated Th0 served as control. Cells were stained at 24h, 48h and 72h and applied to mass cytometry. Percentage of positive cells and mean fluorescence intensity (MFI) of each marker A) CD103, B) CD194, C) CD197, D) CD183, E) CD184, and F) CD38 are shown and compared between iTregs and Th0 controls. The data are represented as mean ± SD for four individual replicates. (*p<0.05, **p<0.01, ***p<0.01).

2.5 Co-stimulatory molecules CD120b and CD137 were upregulated on iTregs

Of the six co-stimulatory molecules analyzed, only two CD120b (TNFR2) and CD137 (TNFRSF9), were found to be significantly upregulated on iTregs compared to Th0 cells. More than 70% of iTreg expressed the surface marker CD137, which was statistically significantly upregulated on iTregs at every timepoint (figure 8B, left panel). In addition, the MFI values were also significantly higher on iTregs than on Th0 cells (figure 8B, right panel).

In comparison, the expression of CD120b varied highly between the individual donors and the percentage of CD120b positive cells was statistically significantly higher on iTregs than on Th0 cells at 48 and 72h (figure 8A, left panel). The MFI values indicated the same pattern being significantly higher on iTregs at 48 and 72h timepoints (figure 8A, right panel).

The expression levels of CD27 and ICOS (CD278) did not show any statistical differences between Th0 and iTreg cells. The expression of CD27 was almost 100 % at all timepoints on both iTregs and Th0 cells (figure 8C, left panel) whereas the MFI values revealed a decrease in CD27 expression in both Th0 and iTregs (figure 8C, right panel). In addition, the MFI values were statistically significantly higher on Th0 cells than on iTregs at 48h timepoint. ICOS, a member of CD28-superfamily co-stimulators, showed no statistically significant difference between Th0 and iTreg positive cells at any given timepoint (24h: 0.35, 48h: $p = 0.05$, 72h: $p = 0.19$) (figure 8D, left panel). However, the MFI values of ICOS showed a statistical upregulation on Th0 cells at 48h timepoint compared to iTreg, whereas big donor variation in ICOS expression was observed (figure 8D, right panel). The overall expression of ICOS (percentage and MFI) decreased on iTregs with time (figure 8D).

The expression level of CD28 (percentage and MFI) was greater on Th0 cells than on iTregs at all timepoints (figure 8E) but a statistically significant difference was observed only at 48h (figure 8E, left and right panel).

Almost 80% of Th0 and iTreg cells expressed the surface marker CD134 (TNFRSF4). Statistically significant differences were detected at 48 and 72h timepoints (figure 8F). At the 48h timepoint the expression level and MFI values were statistically significantly upregulated on Th0 cells compared to iTregs. On the other hand, at the 72h timepoint the expression levels were statistically significantly upregulated on iTregs compared to the Th0 cells, but the difference was not detected at MFI values ($p = 0.09$) (figure 8F).

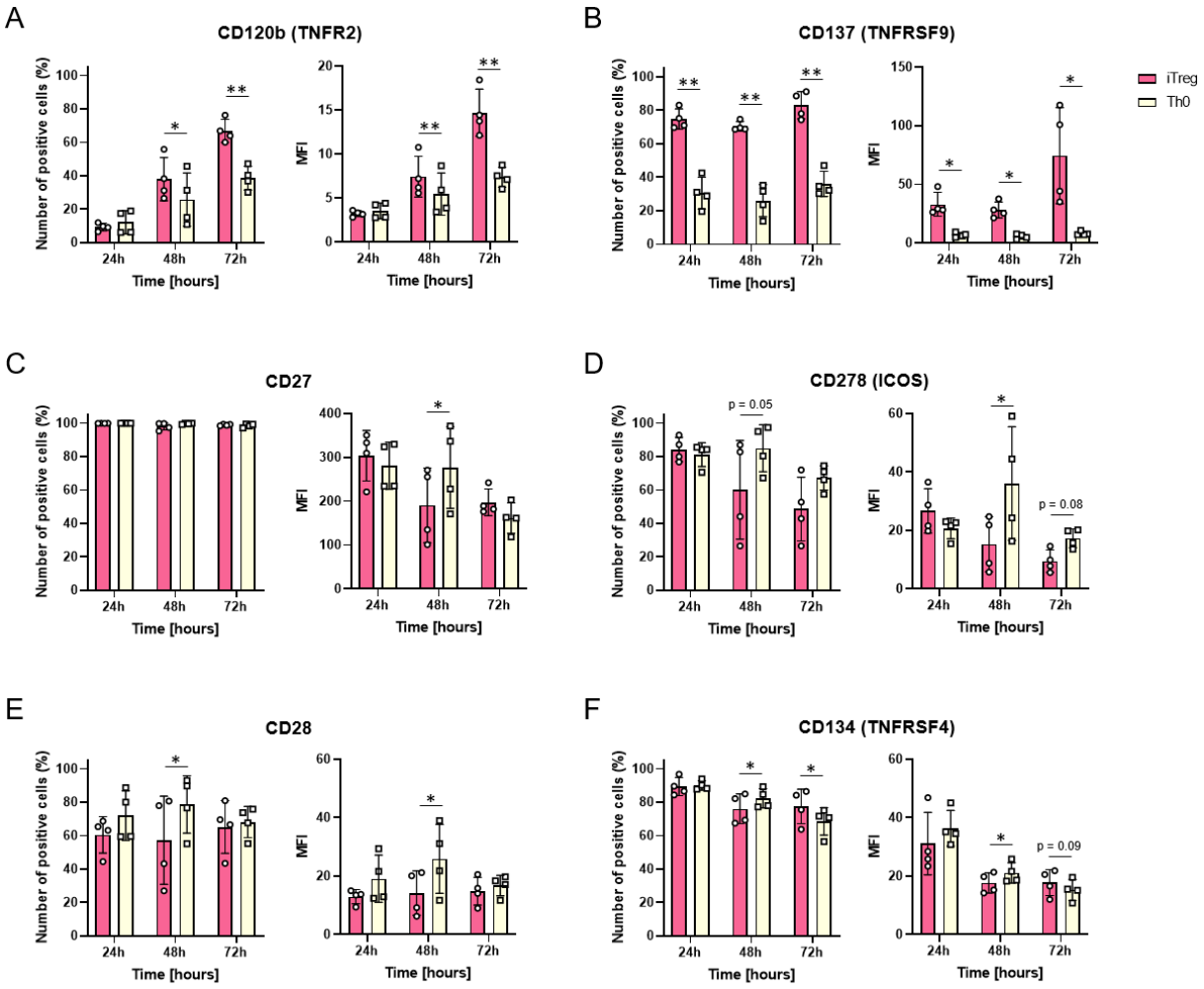


Figure 8. Co-stimulatory molecules CD120b and CD137 were upregulated on iTregs. Naïve $CD4^+$ cells were isolated from 4 individuals and differentiated into iTreg cells. Activated Th0 served as control. Cells were stained at 24h, 48h and 72h and applied to mass cytometry. Percentage of positive cells and mean fluorescence intensity (MFI) of each co-stimulatory marker A) CD120b, B) CD137, C) CD27, D) CD278, E) CD28, and F) CD134 are shown and compared between iTregs and Th0 controls. The data are represented as mean ± SD for four individual replicates. (*p<0.05, **p<0.01).

2.6 HLA-DR, CD15s, CD39 and CD73 were lowly expressed on iTregs

HLA-DR, CD15s, CD39 and CD73 were expressed on a very low level (< 3 % and MFI less than 10) on the iTregs and Th0 cells (figure 9). In addition, the expression levels of some of these molecules decreased over time.

The percentage of HLA-DR positive Th0 and iTreg cells was less than 0.5 % at all timepoints (figure 9A). CD15s was observed to be higher expressed on iTregs at 24 and 72h timepoints, but the percentage of CD15s positive cells was less than 2% and 1%, respectively (figure 9B). The adenosine production mediated by CD39 and CD73 has been linked to immunosuppression

mechanism in Tregs. Less than 2% of CD39 positive Th0 and iTregs were detected (figure 9C), whereas CD73 was expressed on a slightly higher level on iTregs than Th0 cells at all timepoints but percentage of CD73 positive iTreg cells was less than 3% (figure 9D).

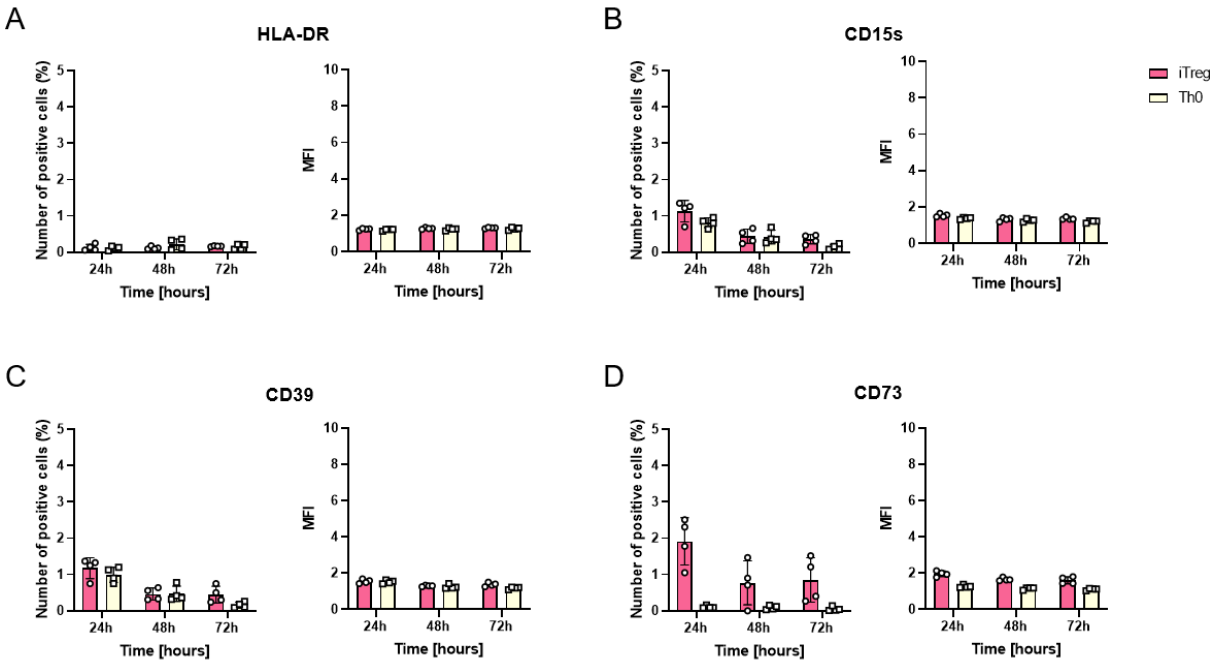


Figure 9. HLA-DR, CD15s, CD39 and CD73 were lowly expressed on iTregs at all timepoints. Naïve $CD4^+$ cells were isolated from 4 individuals and differentiated into iTreg cells. Activated Th0 served as control. Cells were stained at 24h, 48h and 72h and applied to mass cytometry. Percentage of positive cells and mean fluorescence intensity (MFI) of each marker A) HLA-DR, B) CD15s, C) CD39, and D) CD73 are shown. Differences between iTregs and Th0 cells were not significant on the lowly expressed molecules. The data are represented as mean ± SD for four individual replicates.

3 Discussion

Tregs are a distinct Th cell subpopulation and responsible for maintaining the immune homeostasis and self-tolerance by suppressing immune responses. Due to their association in different disease conditions, Tregs have huge potential in clinical applications. However, to use Tregs safely for treatment of cancer and autoimmune disorders their function and features need to be well characterized.

In recent years, mass cytometry has gained popularity to study and characterize different cell types and conditions (Bae et al., 2022; Barcenilla et al., 2019). Mass cytometry is a combination of flow cytometry and mass spectrometry. It can be used to detect more than 40 different cell surface and intracellular markers simultaneously at the single cell level, enabling deep cell phenotyping. Tregs have been studied with a Treg specific panel from *ex vivo* human samples to reveal the complexity of Tregs (Mason et al., 2015) in various diseases, including idiopathic aplastic anemia to predict response to treatment (Kordasti et al., 2016) and type 1 diabetes to study immune cell subset differences between healthy and autoantibody positive children to find new predictive biomarkers (Barcenilla et al., 2019). Moreover, mass cytometry contributes to the identification of diverse Th and Treg subpopulations from human blood, which could be a reference to study abnormalities of different cell populations in immune-mediated pathologies (Kunicki et al., 2018; Olin et al., 2022).

In this study, human induced Treg cells were characterized in-depth using mass cytometry. For this purpose, a panel of 25 markers, characteristic for human Tregs was designed and validated.

$CD4^+$ cells from four individuals were activated and differentiated into control Th0 and iTreg cells, respectively. Moreover, the use of cells from four different donors allows the identification of individual differences in the expression of each marker. Overall, only few individual dependent changes in marker expression were detected in this study, which will be discussed in more detail below.

The present study displays the previous reported Treg phenotype $CD127^{low}$ $CD25^{high}$ $Foxp3^{high}$ (Hartigan-O'Connor et al., 2007; Liu et al., 2006; Santegoets et al., 2015), which has been used as the basis of Treg identification. Here, iTregs gained the Foxp3 expression upon time and had very high and steady CD25 expression (90 ± 10%). The CD127 expression was relatively low on iTregs being less than 5%. Also, the CD45RO memory cell type marker increased upon time, confirming the T cell activation.

Besides the well-known Treg characterization markers, other surface markers were strongly expressed on Tregs when compared to Th0.

CD103, an integrin protein, was strongly upregulated on iTregs (60%) at late differentiation stage while it was not expressed on Th0 control cells, which suggest its functional role on Treg cells. The expression of CD103 is known to be induced by the TGF-β (Hadley et al., 1999; Mokrani et al., 2014; Anz et al., 2011) which would explain its strong upregulation on iTregs compared to Th0 control cells. Moreover, it has also been suggested that CD103 could be a characteristic marker for Treg cells (Allakhverdi et al., 2006; Anz et al., 2011). Previous studies have shown that CD103 mediates cell migration, adhesion, and lymphocyte homing through interactions with E-cadherin (Kim et al., 2019). $CD103^+$ Tregs have shown to express also high levels of GITR, ICOS, CTLA-4, granzyme B, CCR3 and 5 (Lin et al., 2009; Chang et al., 2012) in addition to high Foxp3 expression. Moreover, CD103 has been suggested to have a role in modulating the Foxp3 expression (Braun et al., 2015). However, the relation of CD103 to the suppressive function and phenotype of Tregs is yet to be studied.

Another marker, CCR4 (CD194) has previously been reported to be expressed on Tregs and associated with strong suppressive effector phenotype (Sugiyama et al., 2013). Moreover, CCR4-deficient Tregs lost their suppressive capacity, supporting its functional role in Tregs (Molinaro et al., 2015). In the current study, Tregs also expressed significantly higher amounts of CCR4 compared to Th0 cells, which could be associated with the suppressive capacity of the Tregs. Increased expression of CCR4 on Tregs has been shown in rheumatoid arthritis (Li et al., 2015). The association of $CCR4^+$ Tregs with cancer (Marshall et al., 2020) and autoimmune diseases (Asothai et al., 2015; Li et al., 2015) makes it very interesting target to investigate further. However, further studies are still needed to study the regulation of CCR4 and its role in Treg suppressive function.

Individual dependent variation in marker expression was seen for few markers like CCR4 and CCR7. Both had quite large variability in the expression levels between donors. Although expression of both markers was significantly higher on iTregs than Th0 cells, the percentage of positive cells varied ranging from 0 to 50% on iTregs between donors.

The CD120b has been suggested also as one Treg marker when coexpressed with CD25 (Chen et al., 2010). CD120b is important for TNF signaling in Tregs. TNF-α can induce Treg proliferation through CD120b signaling, which also stabilizes Tregs phenotype and function in

inflammatory environments (Chen et al., 2013). In the current study, the amount of CD120b on iTregs increased upon time and was also significantly upregulated on iTregs compared to Th0 cells. At late timepoint, around 70% of iTregs expressed CD120b, which indicates that CD120b is frequently expressed by Tregs and may be a characteristic marker for these cells. Interestingly, CD120b is shown to enhance the suppressive function of Tregs (Nguyen and Ehrenstein, 2016; He et al., 2018), which in the cancer environment worsens the prognosis and enhances metastasis formation (Chopra et al., 2013). By understanding how CD120b works in Tregs and how it is activated and regulated, it could be considered as a potential new treatment option.

In addition, the co-stimulatory molecule CD137 (TNFRSF9) was highly expressed on iTregs (80%), which is in line with a previous report, describing CD137 as a Treg specific activation marker (Nowak et al., 2018). Further, in that study the identified $CD137^+$ cells were not expressing CD154 (CD40L) which is usually not expressed by Tregs and thus can be used to discriminate Tregs from conventional T cells (Schoenbrunn et al., 2012). Tregs constitutively expressing CD137 have also been demonstrated to be superior in the suppressive capacity compared to Tregs that do not express CD137 (Kachapati et al., 2012). However, due to controversial findings, the role of CD137 in Tregs is under debate (Zhang et al., 2007; Elpek et al., 2007; Akhmetzyanova et al., 2013; Madireddi et al., 2012). CD137 has been shown to be expressed by cancer cells in different malignancies like lymphoma (Ho et al., 2013) and leukemia (Palma et al., 2004), but also tumor-reactive T cells (Zhu and Chen, 2014). Recently it has been reported that Tregs are able to inhibit T cell activity in tumor microenvironment by downregulating the expression of the CD137 ligand (CD137L) on APCs through CD137 trogocytosis (Luu et al., 2021). The functional role of CD137 on Tregs, especially in context of malignancies needs further investigation.

One of the most interesting findings in this study was the significant upregulation of CXCR3 expression on the iTregs. The CXCR3 is commonly associated as marker of Th1 cells (Watanabe et al., 2020) and thus it may indicate a Th1-like phenotype of iTregs. Tregs are known to have different phenotypes that resemble other T helper cells (Duhen et al., 2012). The high CXCR3 expression might be associated with the recruitment of $CXCR3^+$ Tregs to the site of inflammation (Oo et al., 2010). However, as it is known that Tregs can express other Th cells phenotypic markers or even gain other T-helper cell like phenotypes, adding characteristic Th1 or Th17 lineage markers to the panel might give insights in the differentiation status of Tregs.

As previously mentioned, both CD39 and CD73 are important for the adenosine production and thus essential for the adenosine mediated suppression of Tregs (Deaglio et al., 2007). In the current study, the expression levels of CD39 and CD73 remained very low on the iTregs, which might indicate that the adenosine mediated suppression may not be the predominant mechanism. However, in the present study, adenosine levels were not measured. Therefore, further studies are needed to confirm low levels of adenosine, which could be then linked to the suppressive role of Tregs.

Besides, inhibitory molecules have been described to play a major role in the suppressive function of the Tregs. The checkpoint inhibitor PD-1 was expressed by almost 100% of the Tregs at all the studied time points. An increase in LAG-3 and TIM-3 positive iTregs (10%) was observed at 72h, indicating that these molecules might contribute to the suppressive capacity of iTregs at late differentiation stage *in vitro*. However, longer Treg cultures such as 4 or 5 days have to be performed to confirm this hypothesis. CTLA-4 (CD152) is usually upregulated on T cells upon stimulation and acts to suppress T cell activation through DCs by binding its B7 ligands (Oderup et al., 2006). Studies have shown that CTLA-4 is one of the most common inhibitory molecules expressed on Tregs (Takahashi et al., 2000; Tang et al., 2008). In the current study, CTLA-4 expression level stayed relatively low even at the 72h timepoint. Low cell surface expression of CTLA-4 has also been reported previously by other studies and also by another mass cytometry study (Mason et al., 2015). However, it is known that CTLA-4 is constitutively expressed on the surface but also stored intracellularly in high amounts (Takahashi et al., 2000; Jago et al., 2004). Several signaling molecules regulate the CTLA-4 trafficking between intracellular and cell surface compartments (Valk et al., 2008). Therefore, it might be interesting also to investigate the triggers of CLTA-4 trafficking in the current model to better understand its role in suppressive function.

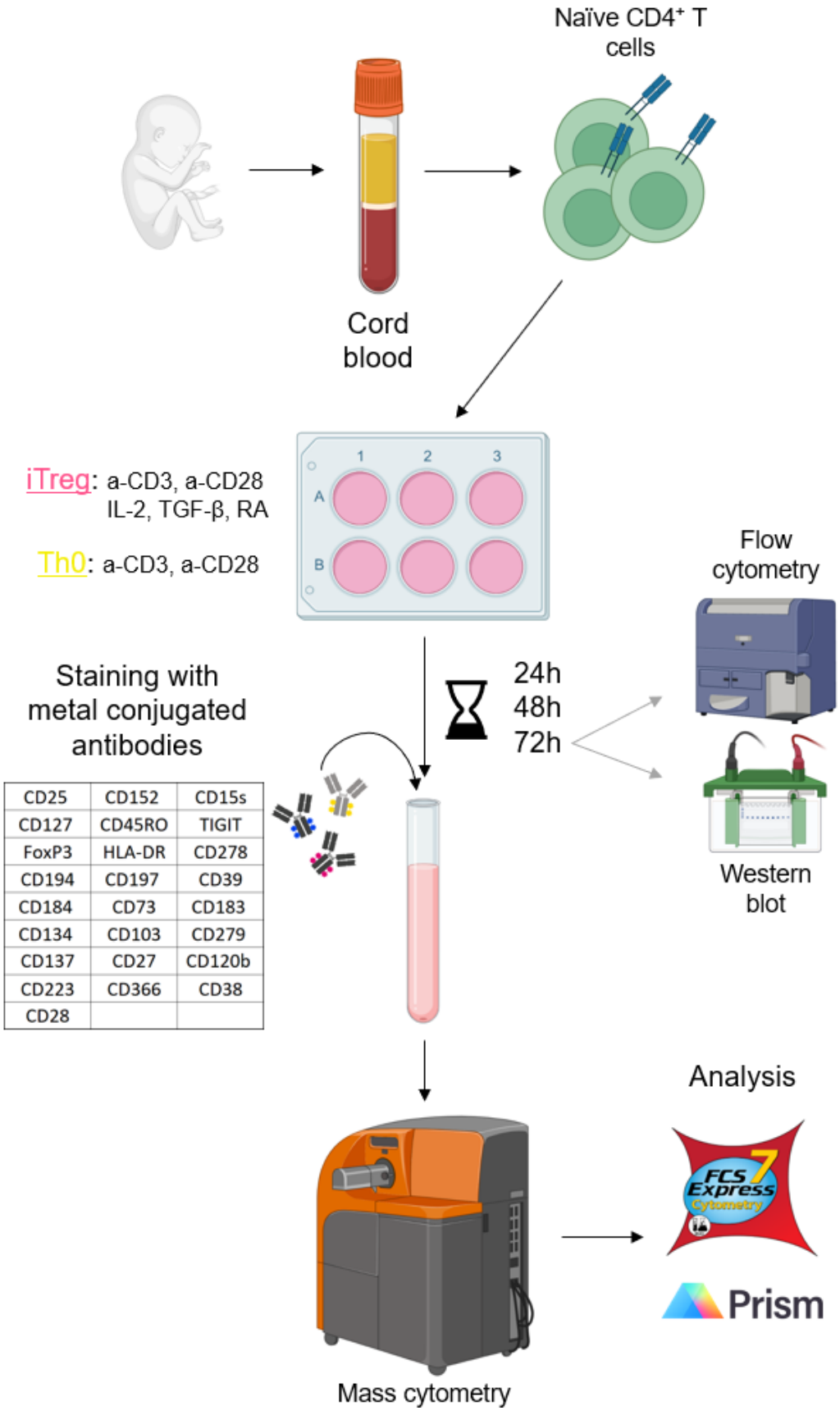
In this study iTregs did not express HLA-DR or CD15s. Both markers have been linked to highly suppressive Tregs (Baecher-Allan et al., 2006; Miyara et al., 2015). Nevertheless, the expression or even upregulation of distinct markers is highly dependent on the microenvironment and disease state, which is difficult to mimic *in vitro*.

In the current study, a Treg panel was successfully established and used for high-dimensional, single-cell characterization of iTregs using mass cytometry. In addition to the characteristic Treg phenotype markers, several inhibitory markers including PD-1, CTLA-4, LAG-3 and TIM-3 were expressed and increased with time on iTreg compared to Th0 cells. Also, some

other surface markers like CD103, CD137, CCR4 and CXCR3 showed a statistically significant upregulation on iTregs, which are interesting targets in context of Treg function. Next, a more detailed cluster analysis will be performed which will give insights in the regulation and cell surface marker expression of human Tregs at single cell level. Moreover, a comprehensive comparison of the marker expression of iTregs, generated in the current study, with natural Tregs purified from human peripheral blood would reveal shared markers, but also highlight the differences between these two Treg populations, which is important to gain deeper insights in Tregs generated *in vitro.*

4 Materials and methods

4.1 Workflow



4.2 $CD4^+$ T cell isolation from umbilical cord blood

Human $CD4^+CD25^-$ T cells were isolated from umbilical cord blood of neonates (Turku University Central Hospital, Turku, Finland) with approval of the Finnish Ethics Committee after oral consent (24.11.1998 article #323). The cord blood was collected in 50 ml tubes supplemented with 1000 IU of heparin (Leo Pharma) by the midwives of the hospital. Fresh blood, not older than two days, was diluted up to 35 ml with Phosphate Buffer Saline (PBS) and layered with Ficoll-Paque Plus (Amersham Bioscience) to isolate the mononuclear cells by density gradient centrifugation (1500 revolutions per minute (rpm), 30 min, no brake). The buffy coat layer was harvested, and washed twice with PBS (1500 rpm, 10 min).

CD4 positive ($CD4^+$) T cells were isolated using magnetic beads (Dynal CD4 Positive Isolation Kit, Invitrogen). In brief, anti-CD4 beads were washed with PBS supplemented with 2 % Fetal Bovine Serum (FBS, Serena) on a magnet stand. The isolated mononuclear cells were incubated with the beads (3.5 µl of beads per ml of undiluted cord blood) in PBS + 2 % FBS on an end-over-end mixer (20 min, +4 °C). After incubation, beads were washed with PBS + 2 % FBS on magnet stand to remove unbound material. To detach the $CD4^+$ cells from the bead, the cell-bead mixture was incubated with Detach antibody in gentle mixing shaker (45 min, room temperature (RT)). $CD4^+$ cells were collected from the supernatant on magnet stand and the beads were washed with PBS + 2 % FBS to collect all the $CD4^+$ cells.

The purity of naive $CD4^+$ T cells from individual donors was characterized by CD45RA (naïve T cell) and CD45RO (memory T cell) expression using flow cytometry (described later in 4.7.1).

4.3 CD25 depletion

Depletion of $CD4^+CD25^+$ T cells was performed to remove $CD25^+$ memory T cells and $CD25^{high}$ nTregs using CD25 Microbeads II kit (Miltenyi Biotec) and LD columns.

The enriched naïve (naivety > 60%) $CD4^+$ cells were washed with sterile-filtered MACS buffer (PBS, 0.5 % bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid (EDTA) pH 7.2) (1500 rpm, 10 min, +4 °C). The cells were then incubated with anti-CD25 microbeads (2 µl of microbeads per 1 x 10^6 cells) for 15 min at +4 °C and then washed with MACS buffer (1500 rpm, 10 min, +4 °C). Up to 1 x 10^8 cells were resuspended in 500 µl of MACS buffer and added to LD column, equilibrated with MACS buffer. The LD column was then rinsed

twice with 2 ml of MACS buffer and all the flow through was collected into one tube. The cells were then washed with PBS (1500 rpm, 10 min, RT), counted on cell counter (Countess II FL, Life Technologies) and resuspended into X-Vivo 15 media (Lonza) for iTreg differentiation.

4.4 Cell culture and iTreg differentiation

Naïve $CD4^+CD25^-$ T cells from single donors were activated in presence of plate-bound anti-CD3 (500 ng/ml) and soluble anti-CD28 (500 ng/ml) in X-vivo 15 serum-free medium (Lonza) supplemented with L-glutamine (2 mM, Biowest), penicillin (50 U) and streptomycin (50 µg/ml) (Penicillin - Streptomycin solution, Biowest) (X-vivo 15 media complete). For iTreg differentiation, the following supplements were added to the medium: IL-2 (12 ng/ml, R&D Systems), TGF-β (10 ng/ml, R&D Systems), all-trans retinoic acid (ATRA, 10 nM, Sigma), and human serum (10 %, Biowest). Control Th0 cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in X-vivo 15 media complete, without adding any cytokines. Both Th0 and iTreg cells were cultured for 24, 48 or 72 hours (h) at 37 °C in 5 % CO_2.

Differentiated iTregs were characterized at day 3 by the expression of Foxp3 protein using immunoblotting and intracellular staining followed by flow cytometry (described later in sections 3.6 and 3.7.1 respectively). Furthermore, the functional suppressive capacity of iTregs was assessed as described in the following section 4.5.

4.5 Suppression assay

The suppressive capacity of iTreg cells was analyzed by co-culturing them for four days with $CD4^+CD25^-$ T cells (responder cells). The responder cells were isolated from peripheral blood mononuclear cells obtained from Finnish Red Cross. Large responder cell patches are stored in -150 °C in freezing medium (90 % fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO)) to reduce the variability between donors. One vial of frozen responder cells (~2 – 5 x 10^6 cells/vial) was thawed quickly in +37 ˚C water bath and washed with 10 ml pre-warmed X-vivo 15 media complete (1500 rpm, 10 min, RT). Cells were resuspended into 1 ml of PBS and stained with 5 µM of CellTrace™ Violet, a proliferation tracing dye (Life Technologies) by adding drop by drop to the cell suspension and incubated 20 min at +37 ˚C water bath. 10 ml of RPMI + 10 % FCS was added to staining solution to quench unbound dye and incubated 5 min on ice. Cells were centrifuged (1500 rpm, 10 min, RT), counted with Luna-FX7 (Logos Biosystems) and resuspended in X-vivo 15 media complete.

For co-cultures, a 96-well plate was coated with anti-CD3 (150 ng/well) and 5 x 10^4 responder cells were added to the coated wells. Responder cells were co-cultured with iTreg or Th0 cells at different ratios (responder cell : iTreg/Th0 ratio 1:1, 1:0.5, 1:0.25) in X-vivo 15 media complete supplemented anti-CD28 (125 ng) for four days at +37 ˚C in 5 % CO_2. As a positive control, 1 x 10^5 responder cells were cultured alone under same conditions as the Th0/iTreg co-cultures. As a negative control, 1 x 10^5 responder cells were cultured without any stimulation in X-vivo 15 complete. On fourth day of the culture, cells were washed twice with FACS I buffer (2 % FBS, 0.01 % natriumazide in PBS) (1900 rpm, 2 min, +4 ˚C), resuspended in FACS I buffer and analyzed by BD LSRFortessa flow cytometer (BD Bioscience). Data was analyzed with FlowJo version 10 software.

4.6 Western blot analyses

Cell pellets were lysed with RIPA buffer (Thermo Fisher Scientific) supplemented with Halt Protease & Phosphatase inhibitor cocktail (Thermo Fisher Scientific) and sonicated (Biorubtor UCD-200) on ice with30 sec on / 30 sec off intervals at high frequency (20 kHz) for 7 min or until the cell suspension was clear. The samples were then centrifuged at 14 000 rpm for 15 min at +4 ˚C and the supernatant was transferred to fresh Eppendorf tube. The protein concentration was determined from the supernatant using the Biorad DC protein assay (Bio-Rad) and Synergy H1 hybrid reader (BioTek; 750 nm absorbance wavelength). 20 ng of protein was mixed with loading dye (330 mM TrisHCl pH 6.8, 33mM sodium dodecyl sulfate (SDS), 6 % β-mercaptoethanol, 170 µM bromophenol blue, 30 % glycerol) and heated at +95 ˚C for 5 min to denature protein.

Protein ladder (PageRuler Plus ladder, Thermo Fisher Scientific, Cat# 26619) and samples were loaded on 4 -20 % gradient gel (MINI-PROTEAN TGX Precast gel, Bio-Rad) and run in LAEMMLI running buffer (190 mM glycine, 25 mM Tris-base, 3.4 mM SDS) at 70 V for 20 min followed by 100 V for 2 - 3 h.

The protein bands were then transferred from the gel onto PVDF membrane (Trans-Blot Turbo Mini Transfer Pack, Rio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad) at 25 V, 1.0 A for 30 min.

After transfer, the membrane was washed once with Tris-buffered saline with 0.1% Tween 20 detergent (TBST) washing buffer (0.15 M NaCl, 0.05 M Tris-HCl, 0.1% Tween-20) on a rolling mixer and then blocked with 10 ml of blocking buffer (5 % BSA in TBST) for 1 h at RT on a

rolling mixer. The rat anti-human Foxp3 monoclonal antibody (Invitrogen, Cat# 14-4776-82), diluted in 1 : 1000 in blocking buffer, was then added to the membrane and incubated overnight at +4 ˚C on a rolling mixer. After incubation, the membrane was washed four times with TBST and the secondary antibody, horseradish peroxidase (HRP)-conjugated polyclonal chicken anti-rat IgG (Novex, Cat# A18733), diluted 1 : 5000 in blocking buffer, was added and incubated for 1 h at RT on a rolling mixer, followed by four washing steps with TBST.

The membranes were developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and visualized on Super RX film (FujiFilm) with 10 sec - 1 min exposure time. The films were developed with X-Ray film processor Curie 60 (AGFA Healthcare).

After the visualization, the housekeeping gene β-actin was used as a loading control. The membrane was incubated in Stripping buffer (25mM Glycine, 1% SDS; 3 min), washed with TBST and incubated with monoclonal anti-β-actin primary antibody (Sigma, Cat# A5441), diluted 1 : 7000 in blocking buffer, for 1 h at RT. After incubation the membrane was washed twice with TBST and the secondary antibody m-IgGκ BP conjugated to HRP (Santa Cruz Biotechnol, Cat# SC-516102), diluted 1 : 7000 in blocking buffer, was added for 30 min and then washed with TBST and developed as described above.

An ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA) was used to determine the intensities of individual protein bands.

4.7 Flow cytometry analyses

4.7.1 Naivety staining

The proportion of naive T cells from the isolated $CD4^+$ cells for each individual donor was determined by the expression of CD45RO (memory marker) and CD45RA (naïve marker) using flow cytometer. Around $1x10^5$ cells were taken from each donor, washed (1900 rpm, 2 min, +4 ˚C) once with FACS I buffer and stained with PE-conjugated CD45RO (BD Bioscience, Cat# 348967), diluted in 1 : 100 in FACS I buffer, or fluorescein isothiocyanate (FITC) -conjugated CD45RA (BD Pharmingen, Cat# 555488), diluted in 1 : 100 in FACS I buffer, for 15 min in dark at +4˚C. Then the cells were washed twice with FACS I buffer and resuspended into 150 µl of FACS I buffer for the flow cytometer (LSRFortessa, BD Bioscience) run. The data was analysed using the FlowJo version 10 software (Tree Star, Ashland, OR, USA).

4.7.2 Intracellular Foxp3 staining

At day 3, approximately 100 000 cells from each Th0 and iTreg culture were transferred into a 96-well U-bottom plate, washed twice (1900 rpm, 2 min, +4 °C) in cold FACS I buffer. Cells were then resuspended in Fixation/Permeabilization working solution (Fixation/Permeabilization Concentrate (eBioscience, Cat# 00-5123-43): Fixation/Permeabilization Diluent (eBioscience, Cat# 00-5223-56), 1 : 4) and incubated for 30 min (+4 °C, dark). After incubation the cells were washed once (1900 rpm, 2 min, +4 °C) in 1x Permeabilization buffer (eBioscience, Cat# 00-8333-56) and stained with PE conjugated anti-human Foxp3 antibody (eBioscience, Cat# 12-4776-42) or with PE rat IgG2ak (BD Pharmingen, Cat# 12-4321-42) isotype control, both diluted in 1 : 50 in Permeabilization buffer, and incubated for 30 min (+4 °C, dark). Then cells were washed twice in 1x Permeabilization buffer (1900 rpm, 2 min, +4 °C) and resuspended in FACS I buffer for flow cytometer (LSRFortessa, BD Biosciences) analysis. Data analysis was performed using FlowJo version 10 software and GraphPad Prism (GraphPad Software, Inc) for statistical analyses.

4.8 Mass cytometer

4.8.1 Antibody labeling

Antibodies used in this study were labeled with either lanthanide or cadmium metals. Cadmium conjugated antibodies were labeled in-house already before I joined the group of Prof Lahesmaa using the Maxpar MCP9 Antibody Labeling Kit (Fluidigim) according to the manufacturer's protocol. The antibody labeling with lanthanide metals was done using The Maxpar X8 Antibody Labeling Kit (Fluidigim) according to the manufacturer's protocol.

In brief, the antibody labeling procedure using lanthanide metals contained the following steps. The polymer was preloaded with lanthanide (Ln) by resuspending a single-use X8 polymer into L-Buffer and 50 mM Ln metal solution was added to the suspension and incubated for 40 min at +37 °C water bath. The antibody was then washed by adding stock antibody to the 50 kDa filter and adjusting the volume in the filter to 400 µl with R-Buffer followed by centrifugation at 12,000 × g for 10 min at RT. After incubation, the polymer was loaded to a 3 kDa filter unit with L-Buffer, mixed and centrifuged at 12,000 × g for 25 min at RT. During the polymer centrifugation the antibody was partially reduced by the 4 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride, MilliporeSigma) solution to the antibody and incubated at +37 °C water bath for 30 min. The polymer was washed again with C-Buffer (12,000 × g, 30

min, RT). At the same time the partially reduced antibody was purified by adding C-Buffer to filter and centrifuged (12,000 × g, 10 min, RT) twice. Afterwards, the partially reduced antibody was conjugated with the lanthanide-loaded polymer by resuspending the Ln-loaded polymer in C-Buffer which was then transferred to the antibody in the 50 kDa filter and incubated for 90 min at +37 °C water bath. After incubation, the metal-conjugated antibody was washed with W-Buffer and centrifuged at 12,000 × g for 10 min. The wash was repeated for four times.

After washing, the antibody yield was determined using a NanoDrop 2000 (Thermo Fisher Scientific) spectrophotometer and the product was centrifuged at 12,000 × g for 10 min to remove the W-Buffer. The conjugated antibody concentration was diluted to 0.5 mg/ml with Antibody Stabilizer PBS (Boca Scientific) supplemented with 0.05 % (w/v) sodium azide (MilliporeSigma). The conjugated antibody was then collected to Protein LoBind tube (Eppendorf) and stored at +4 °C.

The labeled antibodies and metals used in the labeling are presented in the table 1. More info about antibodies is shown in Appendix 1.

Table 1. Labeled antibodies and metal isotopes used in labeling. CD278, CD15s and CD39 were labeled with cadmium isotopes 111, 113 and 116. Antibodies CD152, CD45RO, CD73, CD30 and CD120b were labeled with lanthanides 142 Nd, 145 Nd, 168 Er, 148 Nd and 154 Sm respectively.

Metal	Antibody
111 Cd	CD278 (ICOS)
113 Cd	CD15s
116 Cd	CD39
142 Nd	CD152 (CTLA-4)
145 Nd	CD45RO
168 Er	CD73
154 Sm	CD120b (TNFR2)

4.8.2 Cell antigen stainings

The Maxpar® Nuclear Antigen Staining with Fresh Fix -protocol (Fluidigim) was used for the cell staining and complemented with Cell-ID™ Cisplatin -protocol (Fluidigim) to detect viable cells.

The cell staining consists of six main steps and was performed according to the manufacturing protocol. In brief, the first step includes thawing and counting the cells. Cells stored at −180 ˚C were thawed quickly in a +37 ˚C water bath, washed with pre-warmed RPMI 1640 media

(BioWest) twice (1800 rpm, 10 min, RT) and counted with the cell counter. In the second step the cells were resuspended to 1 x 10^7 cells/ml in RPMI and stained with 5 µM cisplatin for 5 min in RT. The staining was quenched with Maxpar® cell staining buffer (CSB, Fluidigim) using 5X the volume of the staining and then centrifuged (300 x g, 5 min, RT). In the third step, the cells were resuspended to 2 – 6 x 10^7 cells/ml in CSB and stained with the surface antibodies according to right concentrations, incubated for total of 30 min with midpoint vortexing and then washed with CSB (300 x g, 5 min, RT). In the fourth step the cells were incubated for 30 min with Nuclear Antigen Staining Buffer, washed twice (800 x g, 5 min, RT) with Nuclear Antigen Staining Perm, stained with nuclear antigen antibodies for 45 min in RT and washed with Maxpar Nuclear Antigen Staining Perm twice (800 x g, 5 min, RT). In the fifth step the cells were fixed with a fresh 1.6 % formaldehyde solution (1 part 16 % formaldehyde (Thermo Fisher Scientific), 9 parts Maxpar PBS) for 10 minutes at RT and centrifuged (800 x g, 5 min, RT). In the sixth step the cells were stained with 125 nM Cell-ID Intercalator-Ir in Maxpar Fix and Perm Buffer for overnight at +4 ˚C. The samples were stored at -80 ˚C in the Intercalator staining solution until the time of mass cytometry analysis.

To prevent batch-to-batch variability in the antibody concentrations, a master mix of all antibodies was prepared, aliquoted and frozen. The full list of antibodies used in this study is shown in Appendix 1.

4.8.3 Antibody titrations

To find the optimal antibody concentration for cell staining, cells were incubated with four different antibody dilutions including 1:50, 1:100, 1:200 and 1:400. For few antibodies an additional dilution (1:800) was used.

4.8.4 Mass cytometry sample acquisition

To remove the intercalation solution, cells were centrifuged (800 x g, 5 min, RT) and washed with CSB (800 x g, 5 min, RT). Then the cells were resuspended in Maxpar Cell Acquisition Solution (CAS), centrifuged (800 x g, 5 min, RT), re-suspend in CAS and filtered into cell strainer cap tubes. Just prior to data acquisition, 0.1X EQ beads (1 part beads to 9 parts cell suspension) were added to cells. Then the tube was loaded to the mass cytometer and data was acquired.

Around 100,000 cells were collected from each sample during the mass cytometry run. In the data cleaning process, some cells were naturally lost and for the final analysis around 70% of the cells were included.

The mass cytometry data was analysed using the FCS Express 7 software (De Novo Software, USA). Prior to the analysis, the dataset was cleaned by gating out beads, dead cells, and duplets and triplets (figure 10). Live singlet cells were used to determine the percentage of expression and the mean fluorescence intensity (MFI) value for each cell marker. Marker MFI values less than 10 were considered as negative expression.

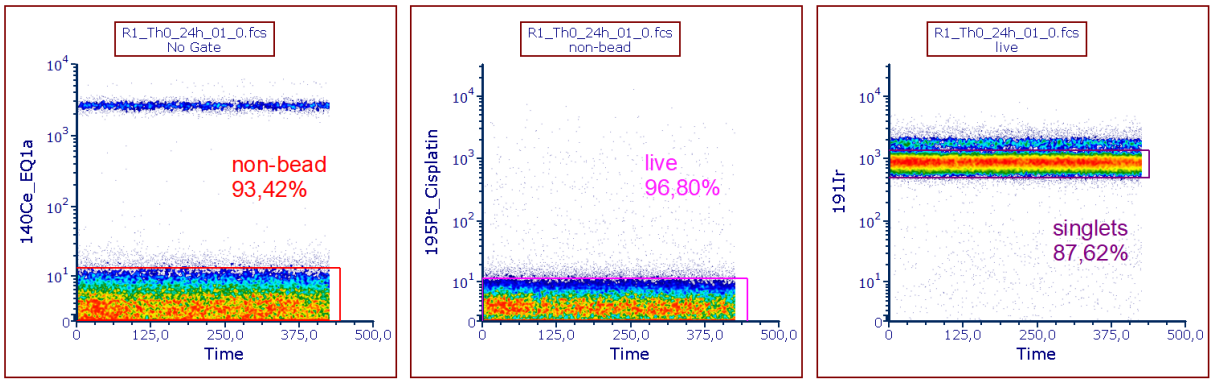


Figure 10. Data cleaning process. In the first step the beads were gated out and those non-beads were used for the second gating of live cells. From live cells, singlet cells were gated and used for the markers' expression analysis. In all gatings time was on x-axis. To get rid of beads, the 140Ce was on y-axis, to detect live cells y-axis was 195Pt-cisplatin and in singlets gating 191Ir was set to y-axis. Gating process is presented from one representative replicate.

4.9 Statistical analyses and data handling

Statistical analyses and figure creations were performed using GraphPad Prism-software (GraphPad Software, San Diego, California, USA).

The statistical significance was tested using paired two-tailed T-test. Statistical significance was concluded when a probability value (p value) was lower than 0.05, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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Abbreviations

APC	Antigen presenting cell
AMP	Adenosine monophosphate
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CNS	Conserver non-coding sequence
CSB	Cell staining buffer
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cell
Foxp3	Forkhead box protein 3
FBS	Fetal bovine serum
GITR	Glucocorticoid-induced TNFR-related protein
h	Hour
ICOS	Inducible T-cell co-stimulator
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
iTreg	Induced regulatory T cell
LAG-3	Lymphocyte-activation gene 3
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NK	Natural killer

PBS	Phosphate Buffer Saline
PD-1	Programmed cell death 1
pTreg	Peripheral regulatory T cell
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TBST	Tris-buffered saline with 0.1% Tween 20 detergent
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
Th0	Activated T cell (without cytokines)
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TNF(R)	Tumor necrosis factor (receptor)
TNFRSF	Tumor necrosis factor receptor superfamily
Treg	Regulatory T cell
tTreg	Thymic regulatory T cell

References

Abbas, A., E. Trotta, D.R. Simeonov, A. Marson, and J.A. Bluestone. 2018. Revisiting IL-2: Biology and therapeutic prospects. *Sci. Immunol.* 3. doi:10.1126/SCIIMMUNOL.AAT1482.

Akhmetzyanova, I., G. Zelinskyy, S. Schimmer, S. Brandau, P. Altenhoff, T. Sparwasser, and U. Dittmer. 2013. Tumor-specific CD4+ T cells develop cytotoxic activity and eliminate virus-induced tumor cells in the absence of regulatory T cells. *Cancer Immunol. Immunother.* 62:257. doi:10.1007/S00262-012-1329-Y.

Allakhverdi, Z., D. Fitzpatrick, A. Boisvert, N. Baba, S. Bouguermouh, M. Sarfati, and G. Delespesse. 2006. Expression of CD103 identifies human regulatory T-cell subsets. *J. Allergy Clin. Immunol.* 118:1342–1349. doi:10.1016/J.JACI.2006.07.034.

Anderson, A.C., N. Joller, and V.K. Kuchroo. 2016. Lag-3, Tim-3, and TIGIT co-inhibitory receptors with specialized functions in immune regulation. *Immunity*. 44:989. doi:10.1016/J.IMMUNI.2016.05.001.

Anz, D., W. Mueller, M. Golic, W.G. Kunz, M. Rapp, V.H. Koelzer, J. Ellermeier, J.W. Ellwart, M. Schnurr, C. Bourquin, and S. Endres. 2011. CD103 is a hallmark of tumor-infiltrating regulatory T cells. *Int. J. cancer*. 129:2417–2426. doi:10.1002/IJC.25902.

Asothai, R., V. Anand, D. Das, P.S. Antil, S. Khandpur, V.K. Sharma, and A. Sharma. 2015. Distinctive Treg associated CCR4-CCL22 expression profile with altered frequency of Th17/Treg cell in the immunopathogenesis of Pemphigus Vulgaris. *Immunobiology*. 220:1129–1135. doi:10.1016/J.IMBIO.2015.06.008.

Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190:995–1003. doi:10.1084/JEM.190.7.995.

Attias, M., T. Al-Aubodah, and C.A. Piccirillo. 2019. Mechanisms of human FoxP3+ Treg cell development and function in health and disease. *Clin. Exp. Immunol.* 197:36–51. doi:10.1111/CEI.13290.

Bae, J., J.E. Kim, H. Perumalsamy, S. Park, Y. Kim, D.W. Jun, and T.H. Yoon. 2022. Mass Cytometry Study on Hepatic Fibrosis and Its Drug-Induced Recovery Using Mouse Peripheral Blood Mononuclear Cells. *Front. Immunol.* 13. doi:10.3389/FIMMU.2022.814030.

Baecher-Allan, C., E. Wolf, and D.A. Hafler. 2006. MHC Class II Expression Identifies Functionally Distinct Human Regulatory T Cells. *J. Immunol.* 176:4622–4631. doi:10.4049/JIMMUNOL.176.8.4622.

Barcenilla, H., L. Åkerman, M. Pihl, J. Ludvigsson, and R. Casas. 2019. Mass Cytometry Identifies Distinct Subsets of Regulatory T Cells and Natural Killer Cells Associated With High Risk for Type 1 Diabetes. *Front. Immunol.* 10. doi:10.3389/FIMMU.2019.00982.

Barzaghi, F., L.C. Amaya Hernandez, B. Neven, S. Ricci, Z.Y. Kucuk, J.J. Bleesing, Z. Nademi, M.A. Slatter, E.R. Ulloa, A. Shcherbina, A. Roppelt, A. Worth, J. Silva, A. Aiuti, L. Murguia-Favela, C. Speckmann, M. Carneiro-Sampaio, J.F. Fernandes, S. Baris, A. Ozen, E. Karakoc-Aydiner, A. Kiykim, A. Schulz, S. Steinmann, L.D. Notarangelo, E. Gambineri, P. Lionetti, W.T. Shearer, L.R. Forbes, C. Martinez, D. Moshous, S. Blanche, A. Fisher, F.M. Ruemmele, C. Tissandier, M. Ouachee-Chardin, F. Rieux-Laucat, M. Cavazzana, W. Qasim, B. Lucarelli, M.H. Albert, I. Kobayashi, L. Alonso, C. Diaz De Heredia, H. Kanegane, A. Lawitschka, J.J. Seo, M. Gonzalez-Vicent, M.A. Diaz, R.K. Goyal, M.G. Sauer, A. Yesilipek, M. Kim, Y. Yilmaz-Demirdag, M. Bhatia, J. Khlevner, E.J. Richmond Padilla, S. Martino, D. Montin, O. Neth, A. Molinos-Quintana, J. Valverde-Fernandez, A. Broides, V. Pinsk, A. Ballauf, F. Haerynck, V. Bordon, C. Dhooge, M.L. Garcia-Lloret, R.G. Bredius, K. Kałwak, E. Haddad, M.G. Seidel, G. Duckers, S.Y. Pai, C.C. Dvorak, S. Ehl, F. Locatelli, F. Goldman, A.R. Gennery, M.J. Cowan, M.G. Roncarolo, and R. Bacchetta. 2018. Long-term follow-up of IPEX syndrome patients after different therapeutic strategies: An international multicenter retrospective study. *J. Allergy Clin. Immunol.* 141:1036-1049.e5. doi:10.1016/J.JACI.2017.10.041.

Beier, K.C., A. Hutloff, A.M. Dittrich, C. Heuck, A. Rauch, K. Büchner, B. Ludewig, H.D. Ochs, H.W. Mages, and R.A. Kroczek. 2000. Induction, binding specificity and function of human ICOS. *Eur. J. Immunol.* 30:3707–17. doi:10.1002/1521-4141.

Bennett, C., J. Christie, F. Ramsdell, M. Brunkow, P. Ferguson, L. Whitesell, T. Kelly, F. Saulsbury, P. Chance, and H. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27:20–21. doi:10.1038/83713.

Bezrodnik, L., M.S. Caldirola, A.G. Seminario, I. Moreira, and M.I. Gaillard. 2014. Follicular bronchiolitis as phenotype associated with CD25 deficiency. *Clin. Exp. Immunol.* 175:227–234. doi:10.1111/CEI.12214.

Bonilla, F.A., and H.C. Oettgen. 2010. Adaptive immunity. *J. Allergy Clin. Immunol.* 125:S33–S40. doi:10.1016/J.JACI.2009.09.017.

Bopp, T., C. Becker, M. Klein, S. Klein-Heßling, A. Palmetshofer, E. Serfling, V. Heib, M. Becker, J. Kubach, S. Schmitt, S. Stoll, H. Schild, M.S. Staege, M. Stassen, H. Jonuleit, and E. Schmitt. 2007. Cyclic adenosine monophosphate is a key component of regulatory T cell–mediated suppression. *J. Exp. Med.* 204:1303–1310. doi:10.1084/JEM.20062129.

Borsellino, G., M. Kleinewietfeld, D. Di Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Höpner, D. Centonze, G. Bernardi, M.L. Dell'Acqua, P.M. Rossini, L. Battistini, O. Rötzschke, and K. Falk. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*. 110:1225–1232. doi:10.1182/BLOOD-2006-12-064527.

Bowakim, N., V. Acolty, M. Dhainaut, H. Yagita, G. Oldenhove, O. Leo, and M. Moser. 2018. Role of the CD27/CD70 pathway in regulatory T cell function. *J. Immunol.* 200.

Le Bras, S., and R.S. Geha. 2006. IPEX and the role of Foxp3 in the development and function of human Tregs. *J. Clin. Invest.* 116:1473. doi:10.1172/JCI28880.

Braun, A., N. Dewert, F. Brunnert, V. Schnabel, J.H. Hardenberg, B. Richter, K. Zachmann, S. Cording, A. Claßen, R. Brans, A. Hamann, J. Huehn, and M.P. Schön. 2015. Integrin αE(CD103) Is Involved in Regulatory T-Cell Function in Allergic Contact Hypersensitivity. *J. Invest. Dermatol.* 135:2982–2991. doi:10.1038/JID.2015.287.

Burmeister, Y., T. Lischke, A.C. Dahler, H.W. Mages, K.-P. Lam, A.J. Coyle, R.A. Kroczek, and A. Hutloff. 2008. ICOS controls the pool size of effector-memory and regulatory T cells. *J. Immunol.* 180:774–782. doi:10.4049/JIMMUNOL.180.2.774.

Cao, X., S.F. Cai, T.A. Fehniger, J. Song, L.I. Collins, D.R. Piwnica-Worms, and T.J. Ley. 2007. Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance. *Immunity*. 27:635–646. doi:10.1016/J.IMMUNI.2007.08.014.

Caudy, A.A., S.T. Reddy, T. Chatila, J.P. Atkinson, and J.W. Verbsky. 2007. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked–like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J. Allergy Clin. Immunol.* 119:482–487. doi:10.1016/J.JACI.2006.10.007.

Cepika, A.M., Y. Sato, J.M.H. Liu, M.J. Uyeda, R. Bacchetta, and M.G. Roncarolo. 2018. Tregopathies: Monogenic diseases resulting in regulatory T-cell deficiency. *J. Allergy Clin. Immunol.* 142:1679–1695. doi:10.1016/J.JACI.2018.10.026.

Chang, L.-Y., Y.-C. Lin, C.-W. Kang, C.-Y. Hsu, Y.-Y. Chu, C.-T. Huang, Y.-J. Day, T.-C. Chen, C.-T. Yeh, and C.-Y. Lin. 2012. The indispensable role of CCR5 for in vivo suppressor function of tumor-derived CD103+ effector/memory regulatory T cells. *J. Immunol.* 189:567–574. doi:10.4049/JIMMUNOL.1200266.

Chen, B.J., J.W. Zhao, D.H. Zhang, A.H. Zheng, and G.Q. Wu. 2022. Immunotherapy of Cancer by Targeting Regulatory T cells. *Int. Immunopharmacol.* 104:108469. doi:10.1016/J.INTIMP.2021.108469.

Chen, X., M. Bäumel, D.N. Männel, O.M.Z. Howard, and J.J. Oppenheim. 2007. Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J. Immunol.* 179:154–161. doi:10.4049/JIMMUNOL.179.1.154.

Chen, X., J.J. Subleski, R. Hamano, O.M.Z. Howard, R.H. Wiltrout, and J.J. Oppenheim. 2010. Co-expression of TNFR2 and CD25 identifies more of the functional CD4+FoxP3+ regulatory T cells in human peripheral blood. *Eur. J. Immunol.* 40:1099. doi:10.1002/EJI.200940022.

Chen, X., X. Wu, Q. Zhou, O.M.Z. Howard, M.G. Netea, and J.J. Oppenheim. 2013. TNFR2 is critical for the stabilization of the CD4+FoxP3+ regulatory T cell phenotype in the inflammatory environment. *J. Immunol.* 190:1076. doi:10.4049/JIMMUNOL.1202659.

Chinen, T., A.K. Kannan, A.G. Levine, X. Fan, U. Klein, Y. Zheng, G. Gasteiger, Y. Feng, J.D. Fontenot, and A.Y. Rudensky. 2016. An essential role for IL-2 receptor in regulatory T cell function. *Nat. Immunol.* 17:1322. doi:10.1038/NI.3540.

Chopra, M., S.S. Riedel, M. Biehl, S. Krieger, V. von Krosigk, C.A. Bäuerlein, C. Brede, A.L.J. Garrote, S. Kraus, V. Schäfer, M. Ritz, K. Mattenheimer, A. Degla, A. Mottok, H. Einsele, H. Wajant, and A. Beilhack. 2013. Tumor necrosis factor receptor 2-dependent homeostasis of regulatory T cells as a player in TNF-induced experimental metastasis. *Carcinogenesis*. 34:1296–1303. doi:10.1093/CARCIN/BGT038.

Claus, C., C. Riether, C. Schürch, M.S. Matter, T. Hilmenyuk, and A.F. Ochsenbein. 2012. CD27 Signaling Increases the Frequency of Regulatory T Cells and Promotes Tumor Growth. *Cancer Res.* 72:3664–3676. doi:10.1158/0008-5472.CAN-11-2791.

Collison, L.W., C.J. Workman, T.T. Kuo, K. Boyd, Y. Wang, K.M. Vignali, R. Cross, D. Sehy, R.S. Blumberg, and D.A.A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 450:566–569. doi:10.1038/NATURE06306.

Courville, E.L., and M.G. Lawrence. 2021. Characteristic CD45RA/CD45RO maturation pattern by flow cytometry associated with the CD45 C77G polymorphism. *Cytom. Part B Clin. Cytom.* 100:602–605. doi:10.1002/CYTO.B.21993.

Cowan, J.E., N.I. McCarthy, and G. Anderson. 2016. CCR7 Controls Thymus Recirculation, but Not Production and Emigration, of Foxp3+ T Cells. *Cell Rep.* 14:1041–1048. doi:10.1016/J.CELREP.2016.01.003.

Croft, M., T. So, W. Duan, and P. Soroosh. 2009. The Significance of OX40 and OX40L to T cell Biology and Immune Disease. *Immunol. Rev.* 229:173. doi:10.1111/J.1600-065X.2009.00766.X.

Curti, A., S. Pandolfi, B. Valzasina, M. Aluigi, A. Isidori, E. Ferri, V. Salvestrini, G. Bonanno, S. Rutella, I. Durelli, A.L. Horenstein, F. Fiore, M. Massaia, M.P. Colombo, M. Baccarani, and R.M. Lemoli. 2007. Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25- into CD25+ T regulatory cells. *Blood*. 109:2871–2877. doi:10.1182/BLOOD-2006-07-036863.

Deaglio, S., K.M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J.F. Chen, K. Enjyoji, J. Linden, M. Oukka, V.K. Kuchroo, T.B. Strom, and S.C. Robson. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* 204:1257–1265. doi:10.1084/JEM.20062512.

Duhen, T., R. Duhen, A. Lanzavecchia, F. Sallusto, and D.J. Campbell. 2012. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood*. 119:4430. doi:10.1182/BLOOD-2011-11-392324.

Eggenhuizen, P.J., B.H. Ng, and J.D. Ooi. 2020. Treg Enhancing Therapies to Treat Autoimmune Diseases. *Int. J. Mol. Sci. 2020, Vol. 21, Page 7015*. 21:7015. doi:10.3390/IJMS21197015.

Elpek, K.G., E.S. Yolcu, D.D.H. Franke, C. Lacelle, R.-H. Schabowsky, and H. Shirwan. 2007. Ex vivo expansion of CD4+CD25+FoxP3+ T regulatory cells based on synergy between IL-2 and 4-1BB signaling. *J. Immunol.* 179:7295–7304. doi:10.4049/JIMMUNOL.179.11.7295.

Fabbri, M., C. Smart, and R. Pardi. 2003. T lymphocytes. *Int. J. Biochem. Cell Biol.* 35:1004–1008. doi:10.1016/S1357-2725(03)00037-2.

Fallarino, F., U. Grohmann, K.W. Hwang, C. Orabona, C. Vacca, R. Bianchi, M.L. Belladonna, M.C. Fioretti, M.L. Alegre, and P. Puccetti. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nat. Immunol.* 4:1206–1212. doi:10.1038/NI1003.

Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol. 2003 44*. 4:330–336. doi:10.1038/ni904.

Fontenot, J.D., J.P. Rasmussen, M.A. Gavin, and A.Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6:1142–1151. doi:10.1038/NI1263.

Francisco, L.M., P.T. Sage, and A.H. Sharpe. 2010. The PD-1 pathway in tolerance and autoimmunity. *Immunol. Rev.* 236:219–242. doi:10.1111/J.1600-065X.2010.00923.X.

Gámez-Díaz, L., D. August, P. Stepensky, S. Revel-Vilk, M.G. Seidel, M. Noriko, T. Morio, A.J.J. Worth, J. Blessing, F. Van De Veerdonk, T. Feuchtinger, M. Kanariou, A. Schmitt-Graeff, S. Jung, S. Seneviratne, S. Burns, B.H. Belohradsky, N. Rezaei, S. Bakhtiar, C. Speckmann, M. Jordan, and B. Grimbacher. 2016. The extended phenotype of LPS-responsive beige-like anchor protein (LRBA) deficiency. *J. Allergy Clin. Immunol.* 137:223–230. doi:10.1016/J.JACI.2015.09.025.

Gao, Y., M. You, J. Fu, M. Tian, X. Zhong, C. Du, Z. Hong, Z. Zhu, J. Liu, G.J. Markowitz, F.S. Wang, and P. Yang. 2022. Intratumoral stem-like CCR4+ regulatory T cells orchestrate the immunosuppressive microenvironment in HCC associated with hepatitis B. *J. Hepatol.* 76:148–159. doi:10.1016/J.JHEP.2021.08.029.

Garín, M.I., N.C. Chu, D. Golshayan, E. Cernuda-Morollón, R. Wait, and R.I. Lechler. 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood*. 109:2058–2065. doi:10.1182/BLOOD-2006-04-016451.

Ghiringhelli, F., C. Ménard, M. Terme, C. Flament, J. Taieb, N. Chaput, P.E. Puig, S. Novault, B. Escudier, E. Vivier, A. Lecesne, C. Robert, J.Y. Blay, J. Bernard, S. Caillat-Zucman, A. Freitas, T. Tursz, O. Wagner-Ballon, C. Capron, W. Vainchencker, F. Martin, and L. Zitvogel. 2005. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J. Exp. Med.* 202:1075–1085. doi:10.1084/JEM.20051511.

Gianchecchi, E., D.V. Delfino, and A. Fierabracci. 2013. Recent insights into the role of the PD-1/PD-L1 pathway in immunological tolerance and autoimmunity. *Autoimmun. Rev.* 12:1091–1100. doi:10.1016/J.AUTREV.2013.05.003.

Gobert, M., I. Treilleux, N. Bendriss-Vermare, T. Bachelot, S. Goddard-Leon, V. Arfl, C. Biota, A.C. Doffin, I. Durand, D. Olive, S. Perez, N. Pasqual, C. Faure, I. Ray-Coquard, A. Puisieux, C. Caux, J.Y. Blay, and C. Ménétrier-Caux. 2009. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates

surrounding primary breast tumors and lead to an adverse clinical utcome. *Cancer Res.* 69:2000–2009. doi:10.1158/0008-5472.CAN-08-2360.

Goswami, T.K., M. Singh, M. Dhawan, S. Mitra, T. Bin Emran, A.A. Rabaan, A. Al Mutair, Z. Al Alawi, S. Alhumaid, and K. Dhama. 2022. Regulatory T cells (Tregs) and their therapeutic potential against autoimmune disorders – Advances and challenges. *https://doi.org/10.1080/21645515.2022.2035117*. 18. doi:10.1080/21645515.2022.2035117.

Gotot, J., C. Gottschalk, S. Leopold, P.A. Knolle, H. Yagita, C. Kurts, and I. Ludwig-Portugall. 2012. Regulatory T cells use programmed death 1 ligands to directly suppress autoreactive B cells in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 109:10468–10473. doi:10.1073/PNAS.1201131109/-/DCSUPPLEMENTAL.

Goudy, K., D. Aydin, F. Barzaghi, E. Gambineri, M. Vignoli, S.C. Mannurita, C. Doglioni, M. Ponzoni, M.P. Cicalese, A. Assanelli, A. Tommasini, I. Brigida, R.M. Dellepiane, S. Martino, S. Olek, A. Aiuti, F. Ciceri, M.G. Roncarolo, and R. Bacchetta. 2013. Human IL2RA null mutation mediates immunodeficiency with lymphoproliferation and autoimmunity. *Clin. Immunol.* 146:248–261. doi:10.1016/J.CLIM.2013.01.004.

Groom, J.R., and A.D. Luster. 2011. CXCR3 in T cell function. *Exp. Cell Res.* 317:620. doi:10.1016/J.YEXCR.2010.12.017.

Grossman, W.J., J.W. Verbsky, W. Barchet, M. Colonna, J.P. Atkinson, and T.J. Ley. 2004a. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity*. 21:589–601. doi:10.1016/J.IMMUNI.2004.09.002.

Grossman, W.J., J.W. Verbsky, B.L. Tollefsen, C. Kemper, J.P. Atkinson, and T.J. Ley. 2004b. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood*. 104:2840–2848. doi:10.1182/BLOOD-2004-03-0859.

Hadley, G.A., E.A. Rostapshova, D.M. Gomolka, B.M. Taylor, S.T. Bartlett, C.I. Drachenberg, and M.R. Weir. 1999. Regulation of the epithelial cell-specific integrin, CD103, by human CD8+ cytolytic T lymphocytes. *Transplantation*. 67:1418–1425. doi:10.1097/00007890-199906150-00005.

Harjunpää, H., and C. Guillerey. 2020. TIGIT as an emerging immune checkpoint. *Clin. Exp. Immunol.* 200:108. doi:10.1111/CEI.13407.

Hartigan-O'Connor, D.J., C. Poon, E. Sinclair, and J.M. McCune. 2007. Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127),

allowing consistent identification and sorting of live cells. *J. Immunol. Methods*. 319:41–52. doi:10.1016/J.JIM.2006.10.008.

Hasegawa, H., A. Inoue, M. Kohno, J. Lei, T. Miyazaki, O. Yoshie, M. Nose, and M. Yasukawa. 2007. Therapeutic effect of CXCR3-expressing regulatory T cells on liver, lung and intestinal damages in a murine acute GVHD model. *Gene Ther. 2008 153*. 15:171–182. doi:10.1038/sj.gt.3303051.

He, T., S. Liu, S. Chen, J. Ye, X. Wu, Z. Bian, and X. Chen. 2018. The p38 MAPK Inhibitor SB203580 Abrogates Tumor Necrosis Factor-Induced Proliferative Expansion of Mouse CD4 + Foxp3 + Regulatory T Cells. *Front. Immunol.* 9. doi:10.3389/FIMMU.2018.01556.

Hendriks, J., L.A. Gravestein, K. Tesselaar, R.A.W. Van Lier, T.N.M. Schumacher, and J. Borst. 2000. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat. Immunol.* 1:433–440. doi:10.1038/80877.

Ho, W.T., W.L. Pang, S.M. Chong, A. Castella, S. Al-Salam, T.E. Tan, M.C. Moh, L.K. Koh, S.U. Gan, C.K. Cheng, and H. Schwarz. 2013. Expression of CD137 on Hodgkin and Reed-Sternberg cells inhibits T-cell activation by eliminating CD137 ligand expression. *Cancer Res.* 73:652–661. doi:10.1158/0008-5472.CAN-12-3849.

Horenstein, A.L., A. Chillemi, G. Zaccarello, S. Bruzzone, V. Quarona, A. Zito, S. Serra, and F. Malavasi. 2013. A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. *https://doi.org/10.4161/onci.26246*. 2. doi:10.4161/ONCI.26246.

Hu, H., I. Djuretic, M.S. Sundrud, and A. Rao. 2007. Transcriptional partners in regulatory T cells: Foxp3, Runx and NFAT. *Trends Immunol.* 28:329–332. doi:10.1016/J.IT.2007.06.006.

Hu, Z., Y. Li, A. Van Nieuwenhuijze, H.J. Selden, A.M. Jarrett, A.G. Sorace, T.E. Yankeelov, A. Liston, and L.I.R. Ehrlich. 2017. CCR7 Modulates the Generation of Thymic Regulatory T Cells by Altering the Composition of the Thymic Dendritic Cell Compartment. *Cell Rep.* 21:168–180. doi:10.1016/J.CELREP.2017.09.016.

Huang, C.T., C.J. Workman, D. Flies, X. Pan, A.L. Marson, G. Zhou, E.L. Hipkiss, S. Ravi, J. Kowalski, H.I. Levitsky, J.D. Powell, D.M. Pardoll, C.G. Drake, and D.A.A. Vignali. 2004. Role of LAG-3 in regulatory T cells. *Immunity*. 21:503–513. doi:10.1016/J.IMMUNI.2004.08.010.

Huard, B., P. Prigent, M. Tournier, D. Bruniquel, and F. Triebel. 1995. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte

activation gene-3 (LAG-3)-Ig fusion proteins. *Eur. J. Immunol.* 25:2718–2721. doi:10.1002/EJI.1830250949.

Hutloff, A., A.M. Dittrich, K.C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R.A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature*. 397:263–266. doi:10.1038/16717.

Jago, C.B., J. Yates, N.O. Saraiva Câmara, R.I. Lechler, and G. Lombardi. 2004. Differential expression of CTLA-4 among T cell subsets. *Clin. Exp. Immunol.* 136:463. doi:10.1111/J.1365-2249.2004.02478.X.

Joller, N., E. Lozano, P.R. Burkett, B. Patel, S. Xiao, C. Zhu, J. Xia, T.G. Tan, E. Sefik, V. Yajnik, A.H. Sharpe, F.J. Quintana, D. Mathis, C. Benoist, D.A. Hafler, and V.K. Kuchroo. 2014. Treg cells expressing the co-inhibitory molecule TIGIT selectively inhibit pro-inflammatory Th1 and Th17 cell responses. *Immunity*. 40:569. doi:10.1016/J.IMMUNI.2014.02.012.

Kachapati, K., D.E. Adams, Y. Wu, C.A. Steward, D.B. Rainbow, L.S. Wicker, R.S. Mittler, and W.M. Ridgway. 2012. The B10 Idd9.3 locus mediates accumulation of functionally superior CD137pos T regulatory cells in the NOD Type 1 diabetes model. *J. Immunol.* 189:5001. doi:10.4049/JIMMUNOL.1101013.

Karin, N. 2010. The multiple faces of CXCL12 (SDF-1α) in the regulation of immunity during health and disease. *J. Leukoc. Biol.* 88:463–473. doi:10.1189/JLB.0909602.

Kim, Y., Y. Shin, and G.H. Kang. 2019. Prognostic significance of CD103+ immune cells in solid tumor: a systemic review and meta-analysis. *Sci. Reports 2019 91*. 9:1–7. doi:10.1038/s41598-019-40527-4.

Kobie, J.J., P.R. Shah, L. Yang, J.A. Rebhahn, D.J. Fowell, and T.R. Mosmann. 2006. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J. Immunol.* 177:6780–6786. doi:10.4049/JIMMUNOL.177.10.6780.

Kordasti, S., B. Costantini, T. Seidl, P.P. Abellan, M.M. Llordella, D. McLornan, K.E. Diggins, A. Kulasekararaj, C. Benfatto, X. Feng, A. Smith, S.A. Mian, R. Melchiotti, E. De Rinaldis, R. Ellis, N. Petrov, G.A.M. Povoleri, S.S. Chung, N.S.B. Thomas, F. Farzaneh, J.M. Irish, S. Heck, N.S. Young, J.C.W. Marsh, and G.J. Mufti. 2016. Deep phenotyping of Tregs identifies an immune signature for idiopathic aplastic anemia and predicts response to treatment. *Blood*. 128:1193. doi:10.1182/BLOOD-2016-03-703702.

Kuehn, H.S., W. Ouyang, B. Lo, E.K. Deenick, J.E. Niemela, D.T. Avery, J.N. Schickel, D.Q. Tran, J. Stoddard, Y. Zhang, D.M. Frucht, B. Dumitriu, P. Scheinberg, L.R. Folio, C.A. Frein, S. Price, C. Koh, T. Heller, C.M. Seroogy, A. Huttenlocher, V.K. Rao, H.C. Su, D. Kleiner, L.D. Notarangelo, Y. Rampertaap, K.N. Olivier, J. McElwee, J. Hughes, S. Pittaluga, J.B. Oliveira, E. Meffre, T.A. Fleisher, S.M. Holland, M.J. Lenardo, S.G. Tangye, and G. Uzel. 2014. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science (80-.).* 345:1623–1627. doi:10.1126/SCIENCE.1255904.

Kumar, B. V., T.J. Connors, and D.L. Farber. 2018. Human T Cell Development, Localization, and Function throughout Life. *Immunity*. 48:202–213. doi:10.1016/J.IMMUNI.2018.01.007.

Kunicki, M.A., L.C. Amaya Hernandez, K.L. Davis, R. Bacchetta, and M.-G. Roncarolo. 2018. Identity and Diversity of Human Peripheral Th and T Regulatory Cells Defined by Single-Cell Mass Cytometry. *J. Immunol.* 200:336–346. doi:10.4049/JIMMUNOL.1701025.

Kurobe, H., C. Liu, T. Ueno, F. Saito, I. Ohigashi, N. Seach, R. Arakaki, Y. Hayashi, T. Kitagawa, M. Lipp, R.L. Boyd, and Y. Takahama. 2006. CCR7-Dependent Cortex-to-Medulla Migration of Positively Selected Thymocytes Is Essential for Establishing Central Tolerance. *Immunity*. 24:165–177. doi:10.1016/J.IMMUNI.2005.12.011.

Lee, J., and B. Min. 2020. LAG-3 expression on Foxp3+ regulatory T cells is critical for controlling homeostasis and autoimmune neuroinflammation. *J. Immunol.* 204.

Lee, W., and G.R. Lee. 2018. Transcriptional regulation and development of regulatory T cells. *Exp. Mol. Med.* 50:e456. doi:10.1038/EMM.2017.313.

Lenschow, D.J., T.L. Walunas, and J.A. Bluestone. 1996. CD28/B7 SYSTEM OF T CELL COSTIMULATION. *http://dx.doi.org/10.1146/annurev.immunol.14.1.233*. 14:233–258. doi:10.1146/ANNUREV.IMMUNOL.14.1.233.

Li, N., W. Wei, F. Yin, M. Chen, T.R. Ma, Q. Wu, J.R. Zhou, S.G. Zheng, and J. Han. 2015. The abnormal expression of CCR4 and CCR6 on Tregs in rheumatoid arthritis. *Int. J. Clin. Exp. Med.* 8:15043.

Liang, J.X., Y. Liang, and W. Gao. 2016. Clinicopathological and prognostic significance of sialyl Lewis X overexpression in patients with cancer: a meta-analysis. *Onco. Targets. Ther.* 9:3113. doi:10.2147/OTT.S102389.

Lin, Y.-C., L.-Y. Chang, C.-T. Huang, H.-M. Peng, A. Dutta, T.-C. Chen, C.-T. Yeh, and C.-Y. Lin. 2009. Effector/Memory but Not Naive Regulatory T Cells Are Responsible for

the Loss of Concomitant Tumor Immunity. *J. Immunol.* 182:6095–6104. doi:10.4049/JIMMUNOL.0803829.

Liu, W., A.L. Putnam, Z. Xu-yu, G.L. Szot, M.R. Lee, S. Zhu, P.A. Gottlieb, P. Kapranov, T.R. Gingeras, B.F. Barbara, C. Clayberger, D.M. Soper, S.F. Ziegler, and J.A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* 203:1701. doi:10.1084/JEM.20060772.

Lo, B., J.M. Fritz, H.C. Su, G. Uzel, M.B. Jordan, and M.J. Lenardo. 2016. CHAI and LATAIE: new genetic diseases of CTLA-4 checkpoint insufficiency. *Blood*. 128:1037–1042. doi:10.1182/BLOOD-2016-04-712612.

Lopes, J.E., T.R. Torgerson, L.A. Schubert, S.D. Anover, E.L. Ocheltree, H.D. Ochs, and S.F. Ziegler. 2006. Analysis of FOXP3 Reveals Multiple Domains Required for Its Function as a Transcriptional Repressor. *J. Immunol.* 177:3133–3142. doi:10.4049/JIMMUNOL.177.5.3133.

Lowther, D.E., B.A. Goods, L.E. Lucca, B.A. Lerner, K. Raddassi, D. van Dijk, A.L. Hernandez, X. Duan, M. Gunel, V. Coric, S. Krishnaswamy, J.C. Love, and D.A. Hafler. 2016. PD-1 marks dysfunctional regulatory T cells in malignant gliomas. *JCI insight*. 1. doi:10.1172/JCI.INSIGHT.85935.

Lu, L., J. Barbi, and F. Pan. 2017. The regulation of immune tolerance by FOXP3. *Nat. Rev. Immunol. 2017 1711*. 17:703–717. doi:10.1038/nri.2017.75.

Luu, K., M.V. Patwardhan, Q. Zeng, S.L. Wickström, A. Lundqvist, and H. Schwarz. 2021. Regulatory T Cells Inhibit T Cell Activity by Downregulating CD137 Ligand via CD137 Trogocytosis. *Cells*. 10:1–11. doi:10.3390/CELLS10020353.

Madireddi, S., R.H. Schabowsky, A.K. Srivastava, R.K. Sharma, E.S. Yolcu, and H. Shirwan. 2012. SA-4-1BBL Costimulation Inhibits Conversion of Conventional CD4+ T Cells into CD4+FoxP3+ T Regulatory Cells by Production of IFN-γ. *PLoS One*. 7. doi:10.1371/JOURNAL.PONE.0042459.

Maeda, S., K. Murakami, A. Inoue, T. Yonezawa, and N. Matsuki. 2019. CCR4 blockade depletes regulatory T cells and prolongs survival in a canine model of bladder cancer. *Cancer Immunol. Res.* 7:1175–1187. doi:10.1158/2326-6066.CIR-18-0751/470784/AM/CCR4-BLOCKADE-DEPLETES-REGULATORY-T-CELLS-AND.

Mandapathil, M., S. Lang, E. Gorelik, and T.L. Whiteside. 2009. Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression. *J. Immunol. Methods*. 346:55. doi:10.1016/J.JIM.2009.05.004.

Marshall, J.S., R. Warrington, W. Watson, and H.L. Kim. 2018. An introduction to immunology and immunopathology. *Allergy, Asthma Clin. Immunol. 2018 142*. 14:1–10. doi:10.1186/S13223-018-0278-1.

Marshall, L.A., S. Marubayashi, A. Jorapur, S. Jacobson, M. Zibinsky, O. Robles, D.X. Hu, J.J. Jackson, D. Pookot, J. Sanchez, M. Brovarney, A. Wadsworth, D. Chian, D. Wustrow, P.D. Kassner, G. Cutler, B. Wong, D.G. Brockstedt, and O. Talay. 2020. Tumors establish resistance to immunotherapy by regulating Treg recruitment via CCR4. *J. Immunother. Cancer*. 8:e000764. doi:10.1136/JITC-2020-000764.

Mason, G.M., K. Lowe, R. Melchiotti, R. Ellis, E. de Rinaldis, M. Peakman, S. Heck, G. Lombardi, and T.I.M. Tree. 2015. Phenotypic Complexity of the Human Regulatory T Cell Compartment Revealed by Mass Cytometry. *J. Immunol.* 195:2030–2037. doi:10.4049/JIMMUNOL.1500703/-/DCSUPPLEMENTAL.

McAdam, A.J., T.T. Chang, A.E. Lumelsky, E.A. Greenfield, V.A. Boussiotis, J.S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V.K. Kuchroo, V. Ling, M. Collins, A.H. Sharpe, and G.J. Freeman. 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J. Immunol.* 165:5035–5040. doi:10.4049/JIMMUNOL.165.9.5035.

McClymont, S.A., A.L. Putnam, M.R. Lee, J.H. Esensten, W. Liu, M.A. Hulme, U. Hoffmüller, U. Baron, S. Olek, J.A. Bluestone, and T.M. Brusko. 2011. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J. Immunol.* 186:3918–3926. doi:10.4049/JIMMUNOL.1003099.

Meiron, M., Y. Zohar, R. Anunu, G. Wildbaum, and N. Karin. 2008. CXCL12 (SDF-1α) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific regulatory T cells. *J. Exp. Med.* 205:2643. doi:10.1084/JEM.20080730.

Miyara, M., D. Chader, E. Sage, D. Sugiyama, H. Nishikawa, D. Bouvry, L. Claër, R. Hingorani, R. Balderas, J. Rohrer, N. Warner, A. Chapelier, D. Valeyre, R. Kannagi, S. Sakaguchi, Z. Amoura, and G. Gorochov. 2015. Sialyl Lewis x (CD15s) identifies highly differentiated and most suppressive FOXP3high regulatory T cells in humans. *Proc. Natl. Acad. Sci. U. S. A.* 112:7225–7230. doi:10.1073/PNAS.1508224112/SUPPL_FILE/PNAS.1508224112.SD01.XLS.

Mokrani, M., J. Klibi, D. Bluteau, G. Bismuth, and F. Mami-Chouaib. 2014. Smad and NFAT Pathways Cooperate To Induce CD103 Expression in Human CD8 T Lymphocytes. *J. Immunol.* 192:2471–2479. doi:10.4049/JIMMUNOL.1302192/-/DCSUPPLEMENTAL.

Molinaro, R., C. Pecli, R.F. Guilherme, J.C. Alves-Filho, F.Q. Cunha, C. Canetti, S.L. Kunkel, M.T. Bozza, and C.F. Benjamim. 2015. CCR4 Controls the Suppressive Effects of Regulatory T Cells on Early and Late Events during Severe Sepsis. *PLoS One*. 10. doi:10.1371/JOURNAL.PONE.0133227.

Morandi, F., I. Airoldi, D. Marimpietri, C. Bracci, A.C. Faini, and R. Gramignoli. 2019. CD38, a Receptor with Multifunctional Activities: From Modulatory Functions on Regulatory Cell Subsets and Extracellular Vesicles, to a Target for Therapeutic Strategies. *Cells 2019, Vol. 8, Page 1527*. 8:1527. doi:10.3390/CELLS8121527.

Muth, S., A. Klaric, M. Radsak, H. Schild, and H.C. Probst. 2021. CD27 expression on Treg cells limits immune responses against tumors. *J. Mol. Med.* 1:1–11. doi:10.1007/S00109-021-02116-9/FIGURES/7.

Myers, A.K., L. Perroni, C. Costigan, and W. Reardon. 2006. Clinical and molecular findings in IPEX syndrome. *Arch. Dis. Child.* 91:63. doi:10.1136/ADC.2005.078287.

Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J. Exp. Med.* 194:629–644. doi:10.1084/JEM.194.5.629.

Nguyen, D.X., and M.R. Ehrenstein. 2016. Anti-TNF drives regulatory T cell expansion by paradoxically promoting membrane TNF-TNF-RII binding in rheumatoid arthritis. *J. Exp. Med.* 213:1241–1253. doi:10.1084/JEM.20151255.

Nowak, A., D. Lock, P. Bacher, T. Hohnstein, K. Vogt, J. Gottfreund, P. Giehr, J.K. Polansky, B. Sawitzki, A. Kaiser, J. Walter, and A. Scheffold. 2018. CD137+CD154- expression as a regulatory T cell (Treg)-specific activation signature for identification and sorting of stable human tregs from in vitro expansion cultures. *Front. Immunol.* 9:199. doi:10.3389/FIMMU.2018.00199/BIBTEX.

Oderup, C., L. Cederbom, A. Makowska, C.M. Cilio, and F. Ivars. 2006. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology*. 118:240–249. doi:10.1111/J.1365-2567.2006.02362.X.

Ohue, Y., and H. Nishikawa. 2019. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Sci.* 110:2080. doi:10.1111/CAS.14069.

Olin, A., N. Acevedo, T. Lakshmikanth, Y. Chen, C. Johansson, J. Alm, A. Scheynius, and P. Brodin. 2022. Longitudinal analyses of development of the immune system during the first five years of life in relation to lifestyle. *Allergy*. 77:1583–1595. doi:10.1111/ALL.15232.

Onishi, Y., Z. Fehervari, T. Yamaguchi, and S. Sakaguchi. 2008. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc. Natl. Acad. Sci. U. S. A.* 105:10113–10118. doi:10.1073/PNAS.0711106105.

Ono, M., H. Yaguchi, N. Ohkura, I. Kitabayashi, Y. Nagamura, T. Nomura, Y. Miyachi, T. Tsukada, and S. Sakaguchi. 2007. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature*. 446:685–689. doi:10.1038/NATURE05673.

Oo, Y.H., C.J. Weston, P.F. Lalor, S.M. Curbishley, D.R. Withers, G.M. Reynolds, S. Shetty, J. Harki, J.C. Shaw, B. Eksteen, S.G. Hubscher, L.S.K. Walker, and D.H. Adams. 2010. Distinct Roles for CCR4 and CXCR3 in the Recruitment and Positioning of Regulatory T Cells in the Inflamed Human Liver. *J. Immunol.* 184:2886–2898. doi:10.4049/JIMMUNOL.0901216.

Ouyang, W., O. Beckett, Q. Ma, J.H. Paik, R.A. Depinho, and M.O. Li. 2010. Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. *Nat. Immunol.* 11:618–627. doi:10.1038/NI.1884.

Ouyang, W., W. Liao, C.T. Luo, N. Yin, M. Huse, M. V. Kim, M. Peng, P. Chan, Q. Ma, Y. Mo, D. Meijer, K. Zhao, A.Y. Rudensky, G. Atwal, M.Q. Zhang, and M.O. Li. 2012. Novel Foxo1-dependent transcriptional programs control T(reg) cell function. *Nature*. 491:554–559. doi:10.1038/NATURE11581.

Palma, C., M. Binaschi, M. Bigioni, C.A. Maggi, and C. Goso. 2004. CD137 and CD137 ligand constitutively coexpressed on human T and B leukemia cells signal proliferation and survival. *Int. J. cancer*. 108:390–398. doi:10.1002/IJC.11574.

Pandiyan, P., L. Zheng, S. Ishihara, J. Reed, and M.J. Lenardo. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation–mediated apoptosis of effector CD4+ T cells. *Nat. Immunol. 2007 812*. 8:1353–1362. doi:10.1038/ni1536.

Patton, D.T., M.D. Wilson, W.C. Rowan, D.R. Soond, and K. Okkenhaug. 2011. The PI3K p110δ regulates expression of CD38 on regulatory T cells. *PLoS One*. 6. doi:10.1371/JOURNAL.PONE.0017359.

Prince, H.E., J. York, and E.R. Jensen. 1992. Phenotypic comparison of the three populations of human lymphocytes defined by CD45RO and CD45RA expression. *Cell. Immunol.* 145:254–262. doi:10.1016/0008-8749(92)90329-N.

Raffin, C., L.T. Vo, and J.A. Bluestone. 2019. Treg cell-based therapies: challenges and perspectives. *Nat. Rev. Immunol. 2019 203*. 20:158–172. doi:10.1038/s41577-019-0232-6.

Ren, X., F. Ye, Z. Jiang, Y. Chu, S. Xiong, and Y. Wang. 2007. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell Death Differ.* 14:2076–2084. doi:10.1038/SJ.CDD.4402220.

Robinson, P.W., S.J. Green, C. Carter, J. Coadwell, and P.J. Kilshaw. 2001. Studies on transcriptional regulation of the mucosal T-cell integrin αEβ7 (CD103). *Immunology*. 103:146–154. doi:10.1046/J.1365-2567.2001.01232.X.

Romano, M., G. Fanelli, C.J. Albany, G. Giganti, and G. Lombardi. 2019. Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. *Front. Immunol.* 10:43. doi:10.3389/FIMMU.2019.00043/BIBTEX.

Rubtsov, Y.P., R. Niec, S. Josefowicz, L. Li, J. Darce, D. Mathis, C. Benoist, and A.Y. Rudensky. 2010. Stability of the regulatory T cell lineage in vivo. *Science*. 329:1667. doi:10.1126/SCIENCE.1191996.

Rudra, D., P. Deroos, A. Chaudhry, R.E. Niec, A. Arvey, R.M. Samstein, C. Leslie, S.A. Shaffer, D.R. Goodlett, and A.Y. Rudensky. 2012. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat. Immunol.* 13:1010. doi:10.1038/NI.2402.

Saban, D.R. 2014. The chemokine receptor CCR7 expressed by dendritic cells: A key player in corneal and ocular surface inflammation. *Ocul. Surf.* 12:87. doi:10.1016/J.JTOS.2013.10.007.

Sakaguchi, S., N. Mikami, J.B. Wing, A. Tanaka, K. Ichiyama, and N. Ohkura. 2020. Regulatory T Cells and Human Disease. *Annu. Rev. Immunol.* 38:541–566. doi:10.1146/annurev-immunol-042718-041717.

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

Sakuishi, K., S.F. Ngiow, J.M. Sullivan, M.W.L. Teng, V.K. Kuchroo, M.J. Smyth, and A.C. Anderson. 2013. TIM3+FOXP3+ regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer. *Oncoimmunology*. 2. doi:10.4161/ONCI.23849.

Santagata, S., M. Napolitano, C. D'Alterio, S. Desicato, S. Di Maro, L. Marinelli, A. Fragale, M. Buoncervello, F. Persico, L. Gabriele, E. Novellino, N. Longo, S. Pignata, S. Perdonà, and S. Scala. 2017. Targeting CXCR4 reverts the suppressive activity of T-regulatory cells in renal cancer. *Oncotarget*. 8:77110. doi:10.18632/ONCOTARGET.20363.

Santegoets, S.J.A.M., E.M. Dijkgraaf, A. Battaglia, P. Beckhove, C.M. Britten, A. Gallimore, A. Godkin, C. Gouttefangeas, T.D. de Gruijl, H.J.P.M. Koenen, A. Scheffold, E.M. Shevach, J. Staats, K. Taskén, T.L. Whiteside, J.R. Kroep, M.J.P. Welters, and S.H. van der Burg. 2015. Monitoring regulatory T cells in clinical samples: consensus on an essential marker set and gating strategy for regulatory T cell analysis by flow cytometry. *Cancer Immunol. Immunother.* 64:1271. doi:10.1007/S00262-015-1729-X.

Schlenner, S.M., B. Weigmann, Q. Ruan, Y. Chen, and H. von Boehmer. 2012. Smad3 binding to the foxp3 enhancer is dispensable for the development of regulatory T cells with the exception of the gut. *J. Exp. Med.* 209:1529–1535. doi:10.1084/JEM.20112646.

Schneider, M.A., J.G. Meingassner, M. Lipp, H.D. Moore, and A. Rot. 2007. CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. *J. Exp. Med.* 204:735–745. doi:10.1084/JEM.20061405.

Schoenbrunn, A., M. Frentsch, S. Kohler, J. Keye, H. Dooms, B. Moewes, J. Dong, C. Loddenkemper, J. Sieper, P. Wu, C. Romagnani, N. Matzmohr, and A. Thiel. 2012. A Converse 4-1BB and CD40 Ligand Expression Pattern Delineates Activated Regulatory T Cells (Treg) and Conventional T Cells Enabling Direct Isolation of Alloantigen-Reactive Natural Foxp3+ Treg. *J. Immunol.* 189:5985–5994. doi:10.4049/JIMMUNOL.1201090.

Schubert, D., C. Bode, R. Kenefeck, T.Z. Hou, J.B. Wing, A. Kennedy, A. Bulashevska, B.S. Petersen, A.A. Schäffer, B.A. Grüning, S. Unger, N. Frede, U. Baumann, T. Witte, R.E. Schmidt, G. Dueckers, T. Niehues, S. Seneviratne, M. Kanariou, C. Speckmann, S. Ehl, A. Rensing-Ehl, K. Warnatz, M. Rakhmanov, R. Thimme, P. Hasselblatt, F. Emmerich, T. Cathomen, R. Backofen, P. Fisch, M. Seidl, A. May, A. Schmitt-Graeff, S. Ikemizu, U. Salzer, A. Franke, S. Sakaguchi, L.S.K. Walker, D.M. Sansom, and B.

Grimbacher. 2014. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *Nat. Med.* 20:1410–1416. doi:10.1038/NM.3746.

Selck, C., and M. Dominguez-Villar. 2021. Antigen-Specific Regulatory T Cell Therapy in Autoimmune Diseases and Transplantation. *Front. Immunol.* 12:1748. doi:10.3389/FIMMU.2021.661875/BIBTEX.

Sharfe, N., H.K. Dadi, M. Shahar, and C.M. Roifman. 1997. Human immune disorder arising from mutation of the α chain of the interleukin-2 receptor. *Proc. Natl. Acad. Sci. U. S. A.* 94:3168–3171. doi:10.1073/PNAS.94.7.3168.

Simonetta, F., A. Chiali, C. Cordier, A. Urrutia, I. Girault, S. Bloquet, C. Tanchot, and C. Bourgeois. 2010. Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells. *Eur. J. Immunol.* 40:2528–2538. doi:10.1002/EJI.201040531.

Smyth, L.A., K. Ratnasothy, J.Y.S. Tsang, D. Boardman, A. Warley, R. Lechler, and G. Lombardi. 2013. CD73 expression on extracellular vesicles derived from CD4+CD25+Foxp3+ T cells contributes to their regulatory function. *Eur. J. Immunol.* 43:2430–2440. doi:10.1002/EJI.201242909.

So, T., and M. Croft. 2007. Cutting Edge: OX40 Inhibits TGF-β- and Antigen-Driven Conversion of Naive CD4 T Cells into CD25 + Foxp3 + T cells . *J. Immunol.* 179:1427–1430. doi:10.4049/jimmunol.179.3.1427.

Sugiyama, D., H. Nishikawa, Y. Maeda, M. Nishioka, A. Tanemura, I. Katayama, S. Ezoe, Y. Kanakura, E. Sato, Y. Fukumori, J. Karbach, E. Jäger, and S. Sakaguchi. 2013. Anti-CCR4 mAb selectively depletes effector-Type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans. *Proc. Natl. Acad. Sci. U. S. A.* 110:17945–17950. doi:10.1073/PNAS.1316796110/SUPPL_FILE/PNAS.201316796SI.PDF.

Tai, X., F. Van Laethem, L. Pobezinsky, T. Guinter, S.O. Sharrow, A. Adams, L. Granger, M. Kruhlak, T. Lindsten, C.B. Thompson, L. Feigenbaum, and A. Singer. 2012. Basis of CTLA-4 function in regulatory and conventional CD4+ T cells. *Blood*. 119:5155. doi:10.1182/BLOOD-2011-11-388918.

Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T.W. Mak, and S. Sakaguchi. 2000. Immunologic Self-Tolerance Maintained by Cd25+Cd4+Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte–Associated Antigen 4. *J. Exp. Med.* 192:303. doi:10.1084/JEM.192.2.303.

Tang, A.L., J.R. Teijaro, M.N. Njau, S.S. Chandran, A. Azimzadeh, S.G. Nadler, D.M. Rothstein, and D.L. Farber. 2008. CTLA4 expression is an indicator and regulator of

steady-state CD4+FoxP3+ T cell homeostasis. *J. Immunol.* 181:1806. doi:10.4049/JIMMUNOL.181.3.1806.

Tang, Q. 2015. Therapeutic Window of Interleukin-2 for Autoimmune Diseases. *Diabetes*. 64:1912. doi:10.2337/DB15-0188.

Thomson, A.W., H.R. Turnquist, and G. Raimondi. 2009. Immunoregulatory Functions of mTOR Inhibition. *Nat. Rev. Immunol.* 9:324. doi:10.1038/NRI2546.

Tiemessen, M.M., A.L. Jagger, H.G. Evans, M.J.C. Van Herwijnen, S. John, and L.S. Taams. 2007. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 104:19446–19451. doi:10.1073/PNAS.0706832104.

Timperi, E., and V. Barnaba. 2021. CD39 Regulation and Functions in T Cells. *Int. J. Mol. Sci.* 22. doi:10.3390/IJMS22158068.

Tivol, E.A., F. Borriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone, and A.H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*. 3:541–547. doi:10.1016/1074-7613(95)90125-6.

Togashi, Y., K. Shitara, and H. Nishikawa. 2019. Regulatory T cells in cancer immunosuppression — implications for anticancer therapy. *Nat. Rev. Clin. Oncol. 2019 166*. 16:356–371. doi:10.1038/s41571-019-0175-7.

Tone, Y., K. Furuuchi, Y. Kojima, M.L. Tykocinski, M.I. Greene, and M. Tone. 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat. Immunol.* 9:194–202. doi:10.1038/NI1549.

Tran, D.Q., D.D. Glass, G. Uzel, D.A. Darnell, C. Spalding, S.M. Holland, and E.M. Shevach. 2009. Analysis of adhesion molecules, target cells and role of interleukin-2 in human FOXP3+ regulatory T cell suppressor function. *J. Immunol.* 182:2929. doi:10.4049/JIMMUNOL.0803827.

Turvey, S.E., and D.H. Broide. 2010. Innate immunity. *J. Allergy Clin. Immunol.* 125. doi:10.1016/j.jaci.2009.07.016.

Valk, E., C.E. Rudd, and H. Schneider. 2008. CTLA-4 trafficking and surface expression. *Trends Immunol.* 29:272. doi:10.1016/J.IT.2008.02.011.

Vinay, D.S., and B.S. Kwon. 2011. 4-1BB signaling beyond T cells. *Cell. Mol. Immunol.* 8:281–284. doi:10.1038/cmi.2010.82.

Vu, M.D., X. Xiao, W. Gao, N. Degauque, M. Chen, A. Kroemer, N. Killeen, N. Ishii, and X.C. Li. 2007. OX40 costimulation turns off Foxp3+ Tregs. *Blood*. 110:2501–2510. doi:10.1182/BLOOD-2007-01-070748.

Watanabe, S., Y. Yamada, and H. Murakami. 2020. Expression of Th1/Th2 cell-related chemokine receptors on CD4 + lymphocytes under physiological conditions. *Int. J. Lab. Hematol.* 42:68–76. doi:10.1111/IJLH.13141.

Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science (80-.).* 322:271–275. doi:10.1126/SCIENCE.1160062/SUPPL_FILE/WING.SOM.PDF.

Wing, K., T. Yamaguchi, and S. Sakaguchi. 2011. Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation. *Trends Immunol.* 32:428–433. doi:10.1016/J.IT.2011.06.002.

Wong, M., A. La Cava, and B.H. Hahn. 2013. Blockade of programmed death-1 in young (New Zealand Black x New Zealand White)F1 mice promotes the suppressive capacity of CD4+ regulatory T cells protecting from lupus-like disease. *J. Immunol.* 190:5402–5410. doi:10.4049/JIMMUNOL.1202382.

Workman, C.J., A.L. Szymczak-Workman, L.W. Collison, M.R. Pillai, and D.A.A. Vignali. 2009. The Development and Function of Regulatory T Cells. *Cell. Mol. Life Sci.* 66:2603. doi:10.1007/S00018-009-0026-2.

Xiao, X., W. Gong, G. Demirci, W. Liu, S. Spoerl, X. Chu, D.K. Bishop, L.A. Turka, and X.C. Li. 2012. New Insights on OX40 in the Control of T Cell Immunity and Immune Tolerance In Vivo. *J. Immunol.* 188:892–901. doi:10.4049/JIMMUNOL.1101373.

Yan, J., Y. Zhang, J.P. Zhang, J. Liang, L. Li, and L. Zheng. 2013. Tim-3 Expression Defines Regulatory T Cells in Human Tumors. *PLoS One*. 8:e58006. doi:10.1371/JOURNAL.PONE.0058006.

Yan, M., N. Jene, D. Byrne, E.K.A. Millar, S.A. O'Toole, C.M. McNeil, G.J. Bates, A.L. Harris, A.H. Banham, R.L. Sutherland, and S.B. Fox. 2011. Recruitment of regulatory T cells is correlated with hypoxia-induced CXCR4 expression, and is associated with poor prognosis in basal-like breast cancers. *Breast Cancer Res.* 13. doi:10.1186/BCR2869.

Yang, Y., M.S. Islam, Y. Hu, and X. Chen. 2021. TNFR2: Role in Cancer Immunology and Immunotherapy. *ImmunoTargets Ther.* 10:103. doi:10.2147/ITT.S255224.

Yoshie, O., and K. Matsushima. 2015. CCR4 and its ligands: from bench to bedside. *Int. Immunol.* 27:11–20. doi:10.1093/INTIMM/DXU079.

Yu, X., K. Harden, L.C. Gonzalez, M. Francesco, E. Chiang, B. Irving, I. Tom, S. Ivelja, C.J. Refino, H. Clark, D. Eaton, and J.L. Grogan. 2009. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat. Immunol.* 10:48–57. doi:10.1038/NI.1674.

Zarek, P.E., C.T. Huang, E.R. Lutz, J. Kowalski, M.R. Horton, J. Linden, C.G. Drake, and J.D. Powell. 2008. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood*. 111:251–259. doi:10.1182/BLOOD-2007-03-081646.

Zhang, P., F. Gao, Q. Wang, X. Wang, F. Zhu, C. Ma, W. Sun, and L. Zhang. 2007. Agonistic anti-4-1BB antibody promotes the expansion of natural regulatory T cells while maintaining Foxp3 expression. *Scand. J. Immunol.* 66:435–440. doi:10.1111/J.1365-3083.2007.01994.X.

Zhao, D.M., A.M. Thornton, R.J. DiPaolo, and E.M. Shevach. 2006. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood*. 107:3925–3932. doi:10.1182/BLOOD-2005-11-4502.

Zhu, Y., and L. Chen. 2014. CD137 as a biomarker for tumor-reactive T cells: Finding gold in the desert. *Clin. Cancer Res.* 20:3–5. doi:10.1158/1078-0432.CCR-13-2573/86257/AM/CD137-AS-A-BIOMARKER-FOR-TUMOR-REACTIVE-T-CELLS.

Zhulai, G., and E. Oleinik. 2022. Targeting regulatory T cells in anti-PD-1/PD-L1 cancer immunotherapy. *Scand. J. Immunol.* 95. doi:10.1111/SJI.13129.

Appendices

Appendix 1. List of antibodies used in mass cytometry

Full list of used antibodies, clones, catalog numbers, metal conjugates and the antibody providers for the study.

Metal conjugate	Antibody	Antibody clone	Company	Catalog
111Cd	CD278 (ICOS)	C398.4A	BioLegend	313502
113Cd	CD15s	CSLEX1	BD Biosciences	551344
116Cd	CD39	A1	BioLegend	328221
142Nd	CD152 (CTLA-4)	14D3	Invitrogen	14-1529-82
143Nd	HLA-DR	L243	Fluidigim	3143013B
145Nd	CD45RO	UCHL1	BioLegend	304239
149Sm	CD25 (IL-2Rα)	2A3	Fluidigim	3149010B
150Nd	CD134 (TNFRSF4)	ACT35	Fluidigim	3150023B
151Eu	CD103 (ITGAE)	Ber-ACT8	Fluidigim	3151011B
154Sm	CD120b (TNFR2)	3G7A02	BioLegend	358402
155Gd	CD27	L128	Fluidigim	3155001B
158Gd	CD194 (CCR4)	L291H4	Fluidigim	3158032A
159Tb	TIGIT	MBSA43	Fluidigim	3159038B
160Gd	CD28	CD28.2	Fluidigim	3160003B
162Dy	Foxp3	PCH101	Fluidigim	3162011A
163Dy	CD183 (CXCR3)	G025H7	Fluidigim	3163004B
167Er	CD197 (CCR7)	G043H7	Fluidigim	3167009A
168Er	CD73	AD2	BioLegend	344002
169Tm	CD366 (TIM-3)	F38-2E2	Fluidigim	3169028B
172Yb	CD38	HIT2	Fluidigim	3172007B
173Yb	CD184 (CXCR4)	12G5	Fluidigim	3173001B
176Yb	CD127 (IL-7Rα)	A019D5	Fluidigim	3176004B
209Bi	CD137 (TNFRSF9)	4B4-1	Fluidigim	3209015B
165Ho	CD223 (LAG-3)	11C3C65	Fluidigim	3165037B
175Lu	CD279 (PD-1)	EH12.2H7	Fluidigim	3175008B