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Foxp3^{+} regulatory T cell induction by murine and human Dendritic cells

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Graz, am 11.6.2015 *Christopher Uschnig, eh*

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"Once the mind has been stretched by a new idea,

it will never again return to its original size"

(Oliver Wendell Holmes Jr)

Zusammenfassung

Dendritische Zellen (DC) sind spezialisierte Antigen-präsentierende Zellen (APC), welche phänotypisch, funktionell, und entwicklungsabhängig verschiedene Zelltypen darstellen. DCs sind dabei wesentlich für die adaptive Immunantwort, sowie für das Hervorrufen und die Aufrechterhaltung der immunologischen Toleranz. Ein Mechanismus durch welchen dendritische Zellen Toleranz regulieren ist durch die Induktion von regulatorischen T Zellen (Treg), die wiederum essentiell für die Regulation der Selbsttoleranz und der Verhinderung von Autoimmunerkrankungen sind. Durch kurzzeitige Exposition mit dem mTOR Inhibitor Rapamycin und Transforming growth factor beta (TGFb) konnten wir induziert-tolerogene (it) DCs erzeugen. Bedingt durch die Vielfältigkeit der verschiedenen DC Zelltypen haben wir fünf unterschiedliche murine DC Zelltypen untersucht, um deren tolerogenes Potential für eine it-DC Therapie in einem Mausmodel für Allergien (Asthma) beurteilen zu können. Des Weiteren wurden fünf human DC Zelltypen mit den gleichen Stimuli wie murine DCs angeregt und auf deren Fähigkeit Tregs *in vitro* zu stimulieren untersucht. Die Untersuchungen der aus Milz isolierten murinen DC Zelltypen ergab Folgendes: (1) Die $CD11c^+$ it-DC Zelltypen ($CD4^+$, $CD8a^+$, $CD11b^+$, triple negative (TN), pDC) waren imstande naive antigenspezifische OT-II Zellen in $Foxp3^+$ $CD25^{hi}$ Tregs zu konvertieren. Im direkten Vergleich war diese Fähigkeit nur sehr eingeschränkt in immunogenen DCs vorhanden; (2) Durch den adoptiven Transfer von Antigen-beladenen it-DCs konnten $Foxp3^+$ Tregs *in vivo* induziert werden; (3) Durch die therapeutische Injektion eines it-DC Zelltypus konnte immunologische Toleranz in einem murinem Asthmamodel induziert werden. Des Weiteren wurden neben murinen DCs auch vier aus Blut isolierte humane DC Zelltypen sowie Monozyten generierte DCs untersucht. Humane $CD16^+$ it-DCs und Monozyten generierte it-DCs waren in verstärktem Maße in der Lage, die Umwandlung von naiven T Zellen in Tregs zu induzieren. In Anbetracht dieser neuen Erkenntnisse schlagen wir it-DCs als mögliche neue zelluläre Therapiestrategie für die Behandlung von T-Zell-vermittelten allergischen Erkrankungen wie allergischem Asthma vor.

Abstract

Dendritic cells (DCs) are specialized antigen-presenting cells composed of phenotypically, functionally, and developmentally diverse subsets. DCs are critical for adaptive immunity, as well as for the induction and maintenance of immune tolerance. One way by which DCs regulate tolerance is through the induction of regulatory T cells (Treg), which are essential for regulating self-tolerance and preventing autoimmune disease. Here, we generated induced-tolerogenic (it) DCs via short-term exposure to the mTOR inhibitor rapamycin and transforming growth factor beta (TGFb). Given the diversity of different DC subsets, we assessed the tolerogenic potential of five different murine subsets to explore the therapeutic potential of it-DC therapy in an allergy model, i.e. allergic asthma. In addition, we applied the same stimuli to five different human subsets in order to assess their potential to induce Tregs *in vitro*. Investigation of murine splenic DC subsets revealed: (1) that $CD11c^+$ it-DC subsets ($CD4^+$, $CD8a^+$, $CD11b^+$, triple negative (TN), pDC) were able to convert antigen-specific naïve OT-II T cells into $Foxp3^+$ $CD25^{hi}$ Tregs, compared to immunogenic DCs; (2) that adoptive transfer of antigen-pulsed it-DCs induced $Foxp3^+$ Tregs *in vivo*; and (3) that the therapeutic injection of one of the it-DC subsets induced tolerance in an murine model of allergic asthma. In addition to murine DCs, we investigated four human blood DC subsets ($CD141^+$, $CD16^+$, $CD1c^+$, pDC), and monocyte-derived DCs. In humans, $CD16^+$ it-DCs and monocyte-derived it-DCs preferentially induced the conversion of naïve T cells into the Tregs. In light of these novel findings, we proposed it-DCs as a potential novel cellular therapeutic strategy for the treatment of T cell-mediated allergic disorders, such as allergic asthma.

1 Introduction

1.1 Dendritic cells

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that regulate adaptive immune responses and tolerance by capturing, processing and presenting self and foreign antigens to T cells. They continuously sample their environment for antigens by endocytosis and present these to T- and B-cells in secondary lymphoid organs. DCs are distributed across various organs and comprise subsets that are phenotypically, functionally, and developmentally diverse. The diversity of DCs is reflected in divergent antigen presentation and cross-presentation, as well as different capacities to induce tolerance and differentiation of T regulatory cells. (Shortman and Liu, 2002; Dauer *et al.*, 2003; Chung *et al.*, 2004; Maldonado and Andrian, 2010)

1.1.1 Dendritic cell development

The vast majority of DCs, as well as monocytes and macrophages originate from a macrophage-DC progenitor (MDP) that is derived from the common myeloid progenitor (CMP). (Doulatov *et al.*, 2010; Fogg *et al.*, 2006) MDP are identified by a unique surface phenotype (Lin^{-1} $cKit^{hi}$ $CD115^{+}$ $CX3CR1^{+}$ $Flt3^{+}$ ($CD135^{+}$)), and differentiate into a DC-restricted common DC progenitor (CDP, (Lin^{-} $cKit^{lo}$ CX_3 $CR1^{+}$ $CD115^{+}$ $Flt3^{+}$), as well as a monocyte progenitor in the bone marrow (BM). (Fogg *et al.*, 2006; Liu *et al.*, 2009). CDPs gives rise to pre-DCs that develop into conventional $CD11c^{+}$ MHC-II^{+} DCs (cDC), and pDCs ($CD11c^{int}$ $B220^{+}$), but not monocytes ($CD11b^{+}$ $CD11c^{low}$). Both migrate from the BM to lymphoid organs and non-lymphoid tissues via the blood. (Liu and Nussenzweig, 2010) The functionally immature common monocyte progenitor (derived from the MPD) is the precursor of monocytes and monocyte-derived macrophages in the BM and spleen (Fig. 1). (Hettinger *et al.*, 2013)

1.1.1.1 pre-DCs

The BM is the primary place for pre-DC expansion, but they exit the BM and enter the blood to further differentiate and divide in spleen and other lymphoid tissues. Pre-cDCs ($CD11c^{+}$ Lin^{-} MHC^{-} $SIRP\alpha^{int}$ $Flt3^{+}$) are restricted to the cDC lineage and can be found in BM (0.2%), blood (0.03%), spleen (0.05%), and lymph nodes (0.03%), although they have a short half-life in blood. BM extracted pre-cDCs that are $CD11c^{-}$ are in a lower developmental stage than $CD11c^{+}$ DCs and may share a single common pathway.

[1] Lin^{-} indicates that cells do not express CD3, CD19, Ter119, NK1.1, or B220 antigens

CD11c$^-$ progress to CD11c$^+$ MHC-class II$^+$ pre-DCs, whereas already CD11c$^+$ pre-DCs directly progress to MHC-class II$^+$ *in vitro*. (Naik *et al.*, 2006; Liu *et al.*, 2009)

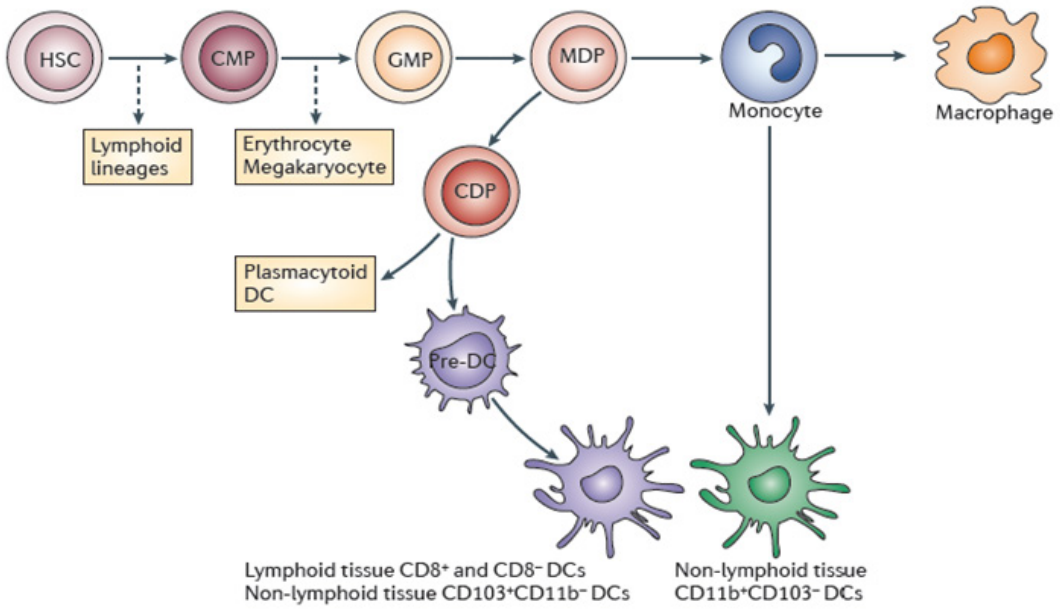

Fig. 1 Differentiation of DCs and monocytes from hematopoietic stem cells (HSC).Common myeloid progenitor (CMP); granulocyte and macrophage progenitor (GMP); DC progenitor (MDP); common DC progenitors (CDPs).Figure edited from (Chow *et al.*, 2011)

CD11c$^-$ MHCII$^-$ (pro DCs) and CD11c$^+$ MHCII$^-$ pre-DCs were able to generate pDCs and cDCs *in vivo*. (Naik *et al.*, 2007) These precursors generate CD8a$^-$ and CD8a$^+$ cDCs as well as pDCs in spleen, though there is a delay of about four days of the peak rise of DC production between the CD11c$^-$ and CD11c$^+$ DC precursors. (Naik *et al.*, 2007) *In vivo* adoptively transferred CD11c$^+$ pre-DCs suggest that contrary to our subset defined as CD11c$^+$ CD8a$^-$ CD4$^-$ CD11b$^-$, the investigated CD11c$^+$ pre-cDC by Kain et al expressed the surface marker CD11b$^+$. Moreover, they were negative for surface expression of MHC class II, CD80 and CD86, markers found on fully developed DCs. (Naik *et al.*, 2007) Remarkably, though CD11c$^+$ pre-cDCs were characterized as CD11b$^+$, adoptively transferred CD11c$^+$ pre-DCs developed into MHC class II$^+$ cDCs of which less than 50% were CD11b$^+$ in spleen after 10 days. Moreover, the vast majority if not all of the pre-DCs developed into CD8a$^+$ (CD11c$^+$ CD8a$^+$ CD4$^-$ CD45RA$^-$) and CD8a$^-$ CD4$^-$ cDCs (CD11c$^+$ CD8a$^-$ CD4$^-$ CD45RA$^-$) with very few CD4$^+$ cDCs.

A study of Ftl3L treated neonatal mice over 7 days revealed that the CD11^{int} CD205$^-$ DCs in control and Flt3L spleens are the biggest and most distinct subpopulation of CD4$^-$ CD8$^-$ DCs (40%) next to a more dispersed population of CD4$^+$CD8$^+$ (about 20%), whereas the CD11c^{hi}/CD205$^+$ subpopulation consists to over 80% of CD8a$^+$ CD4$^-$ DCs. However, the CD11c^{hi} CD205$^-$ DCs show a more distinct CD8$^-$ CD4+ subset. (Vollstedt *et al.*, 2004)

CD205 is a DC maturation marker and higher expressed on matured DCs. (Butler *et al.*, 2007)

Interestingly, $CD11c^-$ and $CD11c^+$ pre-DCs had the morphological appearance of monoblast cells, but $CD11c^-$ pre-DCs lacked the dendritic extensions of the fully formed DCs. Moreover, it was suggested that pDCs and cDCs once differentiated do not change their surface phenotype and are terminally differentiated. (Naik *et al.*, 2007)

Kain et al also identified more immediate cDC precursors in the spleen (0.05% of all splenocytes), defined as $CD11c^{int}$ $CD45RA^{lo}$ $CD43^{int}$ $SIRP^-\alpha^{int}$ $CD4^-$ $CD8^-$ $CD11b^{-/+2}$ $MHCII^-$. These cDC precursors generated $CD11c^{hi}$ $MHCII^{hi}$ DCs and give rise to $CD8^+$ $CD4^-$, $CD8^-$ $CD4^+$ and $CD8^-$ $CD4^-$ cDCs, but not pDCs *in vivo*. This precursor can be divided into three subpopulations of $CD24^{lo}$, $CD24^{int}$, and $CD24^{hi}$ expressing cells from which $CD24^{hi}$ generate almost exclusively $CD8^+$ DCs, $CD24^{int}$ generates $CD8^-$ and $CD8^+$ DCs, and $CD24^{lo}$ mainly $CD8^-$ DCs. (Naik *et al.*, 2006)

Moreover, a $CD11c^{int}$ $CD11b^{hi}$ Mac-3^+ $CD4^{lo}$ $CD8^{lo}$ monocyte-derived DC was identified in the spleen that is preferentially generated and expands in number under inflammatory conditions. (Naik *et al.*, 2006)

1.1.1.2 Flt3L

DCs persistently express fms-like tyrosine kinase 3 (Flt3) during their development. (Liu *et al.*, 2009) Flt3 ligand (Flt3L) is one of the major regulators for DC development, next to GM-CSF and M-CSF, and is maintained through DC development, but also on terminally differentiated DCs. (Schmid *et al.*, 2010) It was shown that in a Flt3L stimulated BM culture, different DC-restricted precursors were generated. Moreover, it was suggested that pDCs and cDCs once differentiated do not change their surface phenotype and are terminally differentiated. (Naik *et al.*, 2007) Flt3L overexpression, such as in Flt3L tumors, in mice cause remarkable $CD11c^+$ DC expansion (up to 44% of all splenocytes) in spleen. (Miller *et al.*, 2003)

1.1.2 DC subsets

DCs that reside solely in lymphoid organs and circulate at low levels in the blood are referred to as lymphoid tissue-resident DCs. Contrary, DCs which are resident in non-lymphoid tissue and migrating to lymph nodes are termed migratory DCs. Lymph tissue resident DCs contain three subsets, the conventional $CD8a^+$ and $CD11b^+$ DC subsets, and plasmacytoid DCs (pDCs). (Crozat *et al.*, 2010) All of these subsets are considered as

[2] -/+ continous negative to low expression.

mature DC populations due to their ability to activate T cells into cell cycle. $CD8a^+$ DCs have the most rapid turnover (36h) of all cDC populations in spleen. (Kamath *et al.*, 2000)

1.1.2.1 conventional DCs (cDCs)

cDCs express high levels of CD11c and MHC class II. Murine pre-cDCs further differentiate into two major subsets that are $CD8a^+$ and $CD4^+$.The spleen contains three subsets, including $CD4^-$ $CD8a^+$ $CD11b^-$ $CD205^+$, $CD4^+$ $CD8a^-$ $CD11b^+$ $CD205^-$ and $CD4^-$ $CD8a^-$ $CD11b^+$ $CD205^-$ cDCs that account for 20%, 40%, and 15% of spleen DCs, respectively. The segregation of DC subsets with the conventional T cell markers CD4 and CD8 is a common used FACS sorting strategy. CD8 on splenic DCs is in the form of an αα-homodimer rather than the typical αβ-heterodimer of T cells. (Vremec *et al.*, 1992; Sato and Fujita, 2007)

1.1.2.2 plasmacytoid DCs (pDCs)

pDCs differ from conventional DCs and are a distinct class of DCs that are present in lymphoid- and non lymphoid tissues. (Liu *et al.*, 2009) pDCs are present in the spleen, but also circulating in the blood. (Kushwah and Hu, 2011) In contrast to cDCs that enter lymph nodes from peripheral tissues, pDCs directly cross the high endothelial venule from the blood through the cell adhesion molecule CD62L. (Nakano *et al.*, 2001) Unlike other DCs, they are able to secrete large amounts of type I interferon in response to viral infections. (Siegal *et al.*, 1999; Swiecki and Colonna, 2010; Ghosh *et al.*, 2010) It remains controversial discussed whether pDCs are unable to phagocytose or micropinocytose exogenous antigens and cross-present antigen. Though, it was reported that antigens complexed with immunoglobulins are internalized via FcRs or lectin receptors and cross presented. pDCs express vast amounts of MHC class II, but the fast turnover does not allow long-lived peptide complexes on the surface for presentation to T cells. (Reizis *et al.*, 2011) It was shown that pDCs mediate oral tolerance and pDC depletion prevented induction of oral tolerance. (Goubier *et al.*, 2008)

1.1.3 DCs in mouse

1.1.3.1 $CD8a^+$ DCs

$CD8a^+$ cDCs are here defined as $CD11c^+$ $CD8a^+$ $CD4^-$ $CD11b^-$ splenic DCs. $CD8a^+$ DCs are highly efficient at cross presenting exogenous antigens to $CD8^+$ T cells on MHC class I. The hallmark functions of mouse $CD8a^+$ cDCs is IL-12p70 secretion and cross-presentation. Moreover, $CD8a^+$ DCs are a major INF-a producer compared with $CD8a^-$ subsets. (Hochrein *et al.*, 2001) IL-12p70 is a heterodimer and the active form of the

proinflammatory cytokine IL-12. IL-12 induces interferon-γ (IFN-γ) production and favors $CD4^+$ T cells to differentiate into type 1 T helper (Th1) cells. (Trinchieri, 2003)

$CD103^+$ cDCs are migratory DCs that carry antigen from peripheral tissues to secondary lymphoid organs (e.g. lymph nodes). It is assumed that the $CD103^+$ cDC subset in peripheral tissues are equivalent to $CD8a^+$ cDCs in lymphoid tissues. (Satpathy *et al.*, 2012)

1.1.3.2 $CD4^+$ DCs

$CD4^+$ DCs are here defined as $CD11c^+$ $CD8a^-$ $CD4^+$ $CD11b^-$ splenic DCs. $CD4^+$ cDCs are considered as more efficient in $CD4^+$ T cell (MHC II restricted) priming than cross-presentation of antigens. (Hochrein *et al.*, 2001) The $CD11b^+$ cDC subset in the periphery is considered equivalent to the $CD4^+$ lymphoid-resident cDC population. (Satpathy *et al.*, 2012)

1.1.3.3 $CD11b^+$ DCs

Splenic $CD8a^-$ $CD4^-$ DCs have the highest capacity to produce IFN-γ. Due to the low abundance of triple negative $CD8a^-$ $CD4^-$ $CD11b^-$ DCs (1%) in normal spleen, the huge majority of cells classified as $CD8a^-$ $CD4^-$ are $CD11b^+$. (Hochrein *et al.*, 2001)

1.1.3.4 pDCs

pDCs express Siglec-H (C-type lectin), BST2 (PDCA1, bone marrow stromal cell antigen 2), and B220. (Zhang *et al.*, 2006; Blasius *et al.*, 2006) pDCs are defined as $CD11c^{int}$ MHC-II^{lo} PDCA-1^{hi} or as $CD11b^-$ Siglec-H^+. The gating on $CD11c^+$ $B220^+$ overlaps with a subset of natural killer cells and it is not accurate to identify pDCs only with these markers. (Blasius *et al.*, 2007) pDCs are specialized in TLR-mediated recognition of viral nucleic acids. pDCs acquire the typical DC morphology after activation. (Swiecki and Colonna, 2010; Ghosh *et al.*, 2010)

1.1.4 DCs in human

In contrast to mouse, studies of human DCs are mainly restricted to blood (monocyte-derived DC protocols) due to the limited availability of human secondary lymphoid organ material. Recent studies showed that the four identified blood and spleen DCs from humans closely resemble each other. (Mittag *et al.*, 2011) DC subsets of mouse and human have similarities, but differ from each other in certain aspects, such as TLR expression, transcription factor expression, and functional properties. (Sallusto and Lanzavecchia, 1994; Mittag *et al.*, 2011)

1.1.4.1 DCs in human blood

Peripheral human blood DCs are identified as CD11c$^+$ HLA-DR$^+$, but don't express CD8a which is the major marker used to segregate mouse cDC subsets. (MacDonald *et al.*, 2002) Functional analysis of human blood DC subsets is generally limited by low cell yields. Human blood contains three major cDC subsets and pDCs (BDCA2$^+$ and BDCA4$^+$). The three cDC subsets are subdivided by the surface markers, CD1c (BDCA1$^+$), CD141 (BDCA3$^+$) and CD16. (Dzionek *et al.*, 2000; Grage-Griebenow *et al.*, 2001)

CD1c$^+$ DCs

CD1c$^+$ DCs may be functional equivalent to murine CD11b$^+$ cDCs. This subset is mostly active in the recognition of extracellular pathogens, the activation of CD4$^+$ T cells and humoral immune responses. (Crozat *et al.*, 2010)

CD141$^+$ and CD16$^+$ DCs

CD141$^+$ human DCs resemble murine CD8a$^+$ cDCs in regard to their gene expression and may be specialized in self-tolerance and in defense against intracellular pathogens. (Crozat *et al.*, 2010) Four studies demonstrated the capacity of human blood CD141$^+$ DC to cross-present and to produce IL-12p70, two functions in which mouse spleen CD8a$^+$ cDCs excel. (Kushwah and Hu, 2011; Robbins *et al.*, 2008) These characteristics are not restricted to CD141$^+$ DCs but are shared by CD1c$^+$ and CD16$^+$ DCs. (Mittag *et al.*, 2011) CD141$^+$ are also reported to have a superior ability to induce Th1 responses compared to CD1c$^+$ DCs and to cross-present viral antigens. (Jongbloed *et al.*, 2010) However, recently it has been shown that peripheral blood CD141$^+$ DCs, considered as the major IL-12 producers, secrete only low amounts of IL-12 in direct comparison with CD1c$^+$ DCs in the presence of various TLR agonists. (Nizzoli *et al.*, 2013)

CD16$^+$ DCs have been described as subset that is also having properties of monocytes and are termed proinflammatory monocytes. CD16$^+$ DCs express high levels of TNF-α , IL-6, and IL-10. CD16$^+$ DCs are similar to CD11b$^+$ CD8$^-$ DCs and murine monocytes due to their high expression of SIRP-α and CD11b, but not CLEC9A. (Mittag *et al.*, 2011)

pDCs

human pDCs are CD11c$^-$ CD123$^+$ (IL-3R$^+$) CD303$^+$ (BDCA2) CD304$^+$ (BDCA4) and have plasma cell-like morphology. CD303 and CD304 are strictly restricted to human pDCs in peripheral blood and bone marrow. (Dzionek *et al.*, 2000; Adam *et al.*, 2005) pDCs are producing large amounts IFN-I in response to viral stimuli, but also seem to be important in antitumor responses. They induce potent antigen-specific T cell responses and also cross-present tumor antigens to cytotoxic T cells. (Tel *et al.*, 2013) Studies implicated that

pDCs have a role in tolerance such as in the engraftment of allogeneic stem cells. However, more factors than just maturation difference is involved in inducing tolerance by pDCs and not fully understood. (Adam *et al.*, 2005)

1.1.4.2 Human monocyte-derived DCs (moDCs)

Monocytes are precursors of macrophages and DCs. (Ziegler-Heitbrock *et al.*, 2010; Palucka *et al.*, 1998) Monocytes, cDCs, and pDCs share the macrophage and DC precursor (MDP) as their common progenitor which is restricted to the BM. It is assumed that monocytes separate in the BM during the transition from MDPs to CDP that represents the cDC and pDC restricted precursor (Fig. 1). (Liu *et al.*, 2009) Monocytes replenish resident macrophages and DCs under steady state and move to sites of infection in response to inflammatory signals. (Swirski *et al.*, 2009)

There are three types of monocytes in human blood; classical ($CD14^{++}$ $CD16^{-}$), intermediate ($CD14^{++}$ $CD16^{+}$), and nonclassical ($CD14^{+}$ $CD16^{++}$)[3]. $CD16^{+}$ monocytes express high levels of MHC class II and TNF-α upon stimulation with TLR ligands. $CD14^{+}$ $CD16^{+}$ monocytes are considered as the proinflammatory subset, but only account for 10% of all monocytes in steady state conditions. (Belge *et al.*, 2002) During an infection or stimulation with macrophage colony-stimulating factor (M-CSF) an increase from classical to intermediate and followed by nonclassical monocytes is observed. (Ziegler-Heitbrock *et al.*, 2010) Monocytes are not only circulating in blood, it was shown that a resident pool of spleen monocytes exists. These splenic resident monocytes exit the spleen in huge amounts upon tissue injury and participate (facilitate) in wound healing and regulation of inflammation. (Swirski *et al.*, 2009)

It is known that monocytes are capable to differentiate into DCs *in vitro* and *in vivo*. The stimulation of $CD14^{+}$ monocytes with macrophage-colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) results in the *in vitro* generation of myeloid (conventional, $CD11c^{+}$) monocyte-derived DCs (moDCs). (Randolph *et al.*, 1999; Romani *et al.*, 1994; Dauer *et al.*, 2003) moDCs are considered as immature DCs, but maturation can be induced by *in vitro* stimulation with LPS. (Sallusto *et al.*, 1999; Sánchez-Torres *et al.*, 2001)

moDC stimulated T cells

A comparison of human $CD14^{+}$ $CD16^{+}$ and $CD14^{+}$ $CD16^{-}$ moDCs revealed that $CD16^{+}$ cells express higher levels of CD86, CD11a, and CD11c compared to $CD16^{-}$ moDCs and induce an increased IL-4 secretion in T cells. Both subsets give rise to DCs. Interestingly,

[3] The $^{+}$ denotes an expression level that is ~ 10-fold and $.^{++}$ is ~ 100-fold above the isotype control

LPS stimulated $CD16^-$ moDCs express higher levels of the proinflammatory cytokine IL-12p70 (Sánchez-Torres *et al.*, 2001), which drives differentiation of $CD4^+$ T cells towards Th1. (Trinchieri, 2003)

In mouse models, it was shown that under inflammatory conditions moDCs are a major producer of IL-12 relative to $CD8a^+$ cDCs *in vivo*, which are considered as a major IL-12 producing cell type. Whereas, $CD8a^+$ IL-12 production can be stimulated through various pathways including MyD88, moDCs IL-12 production is largely dependent on MyD88 signaling. (Zhan *et al.*, 2010) Interestingly, mice that are deficient in the MyD88 pathway show a prolonged survival of skin grafts (McKay *et al.*, 2006) and MyD88 is essential in sustaining mTOR activation. (Chang *et al.*, 2013)

1.2 Regulators of tolerance

Self and non-self discrimination of antigens is a fine balance between maintaining peripheral tolerance to auto-antigens and generate protective immunity to antigens associated with invading pathogens. $CD4^+$ $CD25^+$ $Foxp3^+$ T regulatory cells (Tregs) maintain this balance between homeostatic tolerance and inflammation by bearing a broad TCR diversity that allow them to recognize a wide spectrum of self- and non-self-antigens. (Levings *et al.*, 2006)

1.2.1 T regulatory cells (Tregs)

Regulatory T cells are a distinct subpopulation of T cells that are essential for preventing autoimmune diseases by regulating self-tolerance and homeostasis by down-regulating immune responses to self and exogenous antigens, but also have adverse impact by suppressing sterilizing and antitumor immunity. (Vignali *et al.*, 2008) They are characterized by a high expression of the IL-2 receptor alpha chain (CD25) and Foxp3. (Levings *et al.*, 2006; Relland *et al.*, 2009) Tregs are $CD4^+$ $CD25^+$ and constitute for about 5-10% of peripheral $CD4^+$ T cells.

Treg populations can be divided into two major groups: thymic-derived naturally occurring $CD4^+$ $Foxp3^+$ regulatory T cells (nTregs) and extrathymically derived induced (or adaptive) regulatory T cells (iTregs). (Levings *et al.*, 2006)

1.2.1.1 naturally occurring regulatory T cell (nTreg)

Naturally occurring $CD4^+$ $Foxp3^+$ regulatory T cells (nTregs) differentiate in the normal thymus during lymphocyte development where they need high affinity TCR ligation by an agonist peptide-MHC complex for Foxp3 induction. nTregs represent 1-10% of the human and murine $CD4^+$ T cell population in peripheral blood, thymus, and lymphoid tissues. (Levings *et al.*, 2006; Relland *et al.*, 2009; Curotto Lafaille and Lafaille, 2009)

1.2.1.2 induced tolerogenic regulatory T cell (iTreg)

iTregs arise extrathymically from naïve $CD4^+$ T cells upon antigen activation at peripheral sites under stimulatory conditions such as IL-10 or TGFb-1 with diverse phenotypes. (Levings *et al.*, 2006) One mechanism for iTreg generation from naïve $CD4^+$ T cells under non-inflammatory conditions is antigen presentation by APCs in the absence of maturation signals. (Kretschmer *et al.*, 2005)

Both Treg types differ from their origin, but may work together in many cases. (Levings *et al.*, 2006) All Tregs (including nTregs) express anti-inflammatory cytokines, such as Il-10, TGF-β, or IL-35. (Tang and Bluestone, 2008)

1.2.1.3 Forkhead box p3 (Foxp3)

Foxp3 is an X chromosome encoded transcription factor, largely expressed on $CD4^+$ $CD25^+$ T cells and a master regulator in controlling the development and suppressive functions of Tregs. Foxp3 is essential for the differentiation into Tregs. (Rudensky, 2011) Expression of Foxp3 is detected in 5-10% of mice and 1-5% of human peripheral $CD4^+$ T cells. (Hori *et al.*, 2003)

Over 97% of $Foxp3^+$ cells are $CD4^+$ T cells. In murine lymph nodes over 80% of $CD4^+$ $CD25^{hi}$ cells express Foxp3, whereas only 60% of this population in spleen and 50% in lung tissue. A significant percentage of Foxp3 cells express little or no CD25. Among the $CD4^+$ $Foxp3^+$ population a distinction is made between $CD4^+$ $CD25^{lo/-}$ $Foxp3^+$ and $CD4^+$ $CD25^{hi}$ $Foxp3^+$. It was shown that both populations were able to suppress $CD4^+$ T cell proliferation to the same extent. (Fontenot *et al.*, 2005)

Mice lacking the Foxp3 gene develop the scurfy phenotype. Scurfy mice lose the ability to properly regulate $CD4^+$ T cell activity that results in an aggressive $CD4^+$ and $CD8^+$ T cell mediated autoimmune disease with multi-organ inflammation. Hence, Foxp3 is essential for immune homeostasis. (Brunkow ME *et al.*, 2001) In humans, the loss-of-function mutation leads to multi-organ autoimmune and the inflammatory disorder immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX). (Brunkow ME *et al.*, 2001; Gambineri *et al.*, 2003)

1.2.1.4 Surface markers and expression: nTregs vs. iTregs

The cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a costimulatory T cell receptor (TCR) molecule constitutively expressed on $CD4^+$ $CD25^+$ T cells. CTLA-4 may be required and its blockade abrogates the suppressive function of these Tregs. (Sakaguchi *et al.*, 1995; Takahashi *et al.*, 2000) CTLA-4 is a negative regulator of T cell activation and is crucial for the control of lymphocyte homeostasis by regulating the threshold of signals during T cell activation. (Waterhouse *et al.*, 1995) It reduces the contact time between T cells and APC that further leads to a reduction of proliferation and cytokine secretion of the T cell. (Schneider *et al.*, 2006) $CD4^+$ $CD25^+$ Tregs may require CTLA-4 but not CD28 as a costimulatory molecule for its functional activation. (Takahashi *et al.*, 2000)

It was shown that gene expression profiles and cell surface levels of CD25, CD62L, CD44, and CD103, GITR, Helios, and CTLA-4 of *in vivo* generated iTreg and nTreg cells are very similar, although there is a minimal overlap in the TCR repertoire. Expression of IL-10 and Programmed Death-1 (PD-1) was increased in iTregs and Granzyme B in nTregs. iTregs are essential in regulatory responses and supplement nTregs by enforcing tolerance mechanisms through expanding their TCR diversity. (Haribhai *et al.*, 2011)

Contrary, iTregs produced *in vitro* exhibit a striking different expression profile compared to *in vivo* generated iTregs that much more resemble nTregs in regard to their gene expression pattern. Interestingly, these differences do not affect the suppressive effect, but may affect phenotypic stability of Foxp3 and the phenotype. (Haribhai *et al.*, 2011) *In vitro*, T cells are exposed to a limited set of stimuli (TCR signaling, TGFb, and IL-2), whereas the cells are confronted with a much broader set of cues *in vivo*. (Kretschmer *et al.*, 2005)

	nTreg*	**iTreg***
site of induction	thymus	lymph nodes, spleen, inflamed tissue
costimulation	CD28	CTLA-4
cytokine requirement	IL-2, TGFb?	IL-2, TGFb
common markers	Foxp3, CD25, GITR, CTLA-4, CD39,	Foxp3, CD25, huge variability
specifity	self-antigens	self- and foreign antigens (e.g. allergens)
specific markers	Helios	

Tab. 1 nTreg and iTreg overview. (Schmetterer *et al.*, 2012; Curotto Lafaille and Lafaille, 2009) Helios is expressed in the majority of $CD4^+$ $Foxp3^+$ human PBMCs (human peripheral blood mononuclear cells), and in Tregs from spleen and thymus in mice. Contrary to Foxp3, the absence of Helios does not affect the suppressive capacity of Tregs. (Thornton *et al.*, 2010; Schmetterer *et al.*, 2012; Curotto Lafaille and Lafaille, 2009) *in human and mouse.

1.2.1.5 Treg functions

One of the main functions of Tregs is to suppress autoreactive T cells that escaped from thymic negative selection. Eradication or functional abrogation of Tregs results in the development of diverse autoimmune diseases in human and mouse. (Groux *et al.*, 1997; Maloy and Powrie, 2001; Levings *et al.*, 2006) $Foxp3^+$ Tregs may be responsible for the suppression of self-reactive T cells *in vivo*. (Kim *et al.*, 2007)

1.2.1.6 Treg stimulation: spleen DCs vs. APCs

It was shown that spleen DCs are over 100-fold more potent than DC-depleted splenic APCs ($CD11c^-$) in inducing antigen-specific $CD4^+$ $Foxp3^+$ Tregs from naïve $CD4^+$ T cells. DCs also need much lower (10x) dose of antigen. IL-2 is an essential cytokine for Treg expansion and is provided endogenously through T cells that are stimulated by DCs, but not other APCs. (Yamazaki *et al.*, 2007)

1.2.2 Understanding DC and Treg interactions

Understanding the role of DC subsets in Treg activation is critical for the development of a therapeutic strategy against autoimmune disease.

1.2.2.1 DC recruitment

DC precursors are recruited from the blood into the tissue by a multistep adhesion cascade that involves at least three consecutive steps: tethering and rolling mediated by primary adhesion molecules (selectins), exposure to chemotactic stimulus that leads to conformational activation of integrins (e.g. LFA-1, VLA-4, Mac-1), and arrest mediated by the binding of activated integrins to various ligands (i.e. ICAM-1 (ligand for LFA-1 and Mac-1), ICAM-2 (ligand for LFA-1), VCAM-1 (ligand for VLA-4). (Springer, 1994; Andrian and Mackay, 2000)

1.2.2.2 DC maturation

Tissue-resident DCs are considered as immature with poorly immunogenic properties due to low expression of MHC and little or no costimulatory molecules. Immunological maturation of DCs, the ability to prime naïve T cells, is triggered by detection of non-self exogenous antigens (e.g. bacteria antigens such as LPS) on specific receptors (PAMPs) or tissue distress that is recognized with damage associated molecular patterns (DAMPs). (Maldonado and Andrian, 2010) These stimuli are often referred to as “danger signals”. (Matzinger, 2002)

During maturation, the antigen processing ability of DCs decrease, but the antigen bearing DCs upregulate the chemokine receptor CCR7 (chemokine ligands are CCL19 and CCL21), MHC-II, costimulatory molecules such as CD40, CD80, CD86, as well as cytokines that promote and modulate inflammation, including IL-1β, IL-2, IL-6, IL-8, IL-12, and IL-18. (Banchereau *et al.*, 2000; Alvarez *et al.*, 2008) CCR7 enables DCs to enter lymph vessels and migration to local draining lymph nodes and is a marker for DC maturation. A small fraction of DCs enter the venous blood circulation and deliver the captured antigen to the spleen, BM, and thymus. (Alvarez *et al.*, 2008)

1.2.2.3 Interplay between DCs and Tregs

Recently, a regulatory feedback mechanism between Tregs and DCs was discovered that maintains Treg homeostasis *in vivo*. It was shown that the depletion of Tregs leads to a compensatory Flt3-dependent increase in DCs. Furthermore, a loss of DCs consequently causes a loss of Tregs accompanied by increasing levels of proinflammatory cytokines such as IL-17 and INF-γ. Increasing numbers of DCs also raise the number of Tregs. (Darrasse-Jèze *et al.*, 2009) The ablation of DCs (cDCs, pDCs, and Langerhans cells) under steady-state conditions results in spontaneous autoimmunity with characteristics such as high numbers of Th1 and Th17 cells, neutrophilia, and infiltration of $CD4^+$ T cells into peripheral tissues. (Ohnmacht *et al.*, 2009)

A disruption of the TGFb receptor on DCs impairs the tolerogenic function of DCs resulting in augmented T cell responses and autoimmunity. (Laouar *et al.*, 2008) TGFb is able to induce Foxp3 expression in TCR challenged $CD4^+$ $CD25^-$ naïve T cells in murine models and human *in vitro*. (Chen *et al.*, 2003) The conversion from naïve peripheral and natural (thymic) T cells also occurs *in vivo*. (Chen and Konkel, 2010) TGFb is necessary for $CD4^+$ $Foxp3^+$ Treg induction. (Gabryšová *et al.*, 2011; Kretschmer *et al.*, 2005)

1.2.2.4 Treg induction by DCs

Immature DCs are major inducer of Tregs and suppressors of proliferation of conventional $CD4^+$ T cells in a cell-contact dependent manner *in vivo*. (Mahnke *et al.*, 2003) Immature DCs ($CD83^-$) are derived from human blood monocytes after 6 days of cultivation in the presence of GM-CSF and IL-4. These immature DCs, compared to maturated DCs that were generated from this exact same population through the addition of a cytokine cocktail, showed superior capacity to induce $CD4^+$ $CD25^+$ $CTLA4^+$ regulatory T cells from naïve $CD4^+$ T cells. (Jonuleit *et al.*, 2000)

In contrast, maturate DCs induced a strong differentiation of naïve $CD4^+$ T cells into Th1 cells that express high levels of the proinflammatory cytokines IFN-γ and IL-2, but not IL-4, IL-5, or IL-10. Tregs induced by immature DCs exhibit low proliferation capacity and show a characteristic Treg cytokine profile such as high levels of the immunosuppressive cytokine IL-10 and no or very low secretion of IFN-γ, IL-2, IL-4, or IL-5. IL-2 and IL-4 promote T cell growth. Moreover, they inhibit, in a cell contact dependent manner, the proliferation of cocultured Th1 cells. The antibody-mediated blocking of CTLA-4, IL-10 and TGFb on $CD4^+$ $CD25^+$ $CTLA4^+$ Tregs could not reverse the inhibitory effect on Th1 cells. High doses of IL-2 (100 U/ml) partially restored Th1 expansion. (Jonuleit *et al.*, 2000)

1.2.3 Induced tolerogenesis

1.2.3.1 Tolerogenic DCs (tDCs)

tDCs are defined as DCs that induce and/or enhance tolerance rather than effector responses. Tissue-resident DCs are considered as a population of typically tolerogenic DCs due to their immature developmental stage, although maturate DCs may retain tolerogenic function under certain conditions. tDC that occur naturally and constitutively from hematopoietic precursors are referred to as natural tolerogenic DCs (ntDCs). tDCs which receive signals from their environment, such as chemical agents (e.g. rapamycin) are called induced tolerogenic DCs (itDCs). The potent activity of tDCs and the *in vitro* generation of itDCs with pharmacological agents makes them attractive for immune therapy. However, the role of the different tDC subsets and their capability to induce Tregs is less well understood. (Maldonado and Andrian, 2010) Central tolerance is well understood and involves the deletion of self-reactive T cells by bone marrow-derived DCs in the thymus. (Vandekerckhove *et al.*, 1992) The induction of Tregs and their suppressive capacity is crucial in inducing peripheral tolerance. (Roncarolo *et al.*, 2001) Immunosuppressive drugs targeting key functions of DCs can modulate DC function and development. (Hackstein and Thomson, 2004)

1.2.3.2 Rapamycin

Rapamycin, also known as sirolimus, is a 31-membered macrocyclic lactone ($C_{51}H_{79}NO_{13}$) with a molecular weight of 914.2 and produced from the soil bacterium Streptomyces hygroscopicus. (Gregory *et al.*, 2004) It is used clinically as an immunosuppressive drug to control transplant rejection. (Augustine *et al.*, 2007) Rapamycin affects metabolism and immunogenicity of DCs by inhibiting the serine/threonine kinase mammalian target of rapamycin (mTOR). It has been shown to expand selectively the murine and human $CD4^+$ $CD25^+$ $Foxp3^+$ population *in vitro* and *in vivo* accompanied with suppression of proliferation of $CD4^+$ and $CD8^+$ T effector cells. (Battaglia *et al.*, 2005; Battaglia *et al.*, 2006; Keever-Taylor *et al.*, 2007, Kang *et al.*, 2008) The prolonged treatment with rapamycin also induces the generation of Tregs in the thymus. (Coenen *et al.*, 2007) The complete inhibition of mTOR *in vivo* (conditional knockout mice) results in Treg accumulation upon T cell stimulation and an incapability of mTOR-deficient T cells to differentiate into T helper type 1 (Th1), T helper type 2 (Th2), or T helper type 17 cells. (Delgoffe *et al.*, 2009) It is suggested that rapamycin increases the sensitivity of naïve T cells to TGFb and Treg differentiation also occurs with low levels of TGFb. A complete blockade with neutralizing antibodies against TGFb results in a massive Foxp3 inhibition independent of anti-CD3/anti-CD28 stimulatory intensity. (Gabryšová *et al.*, 2011)

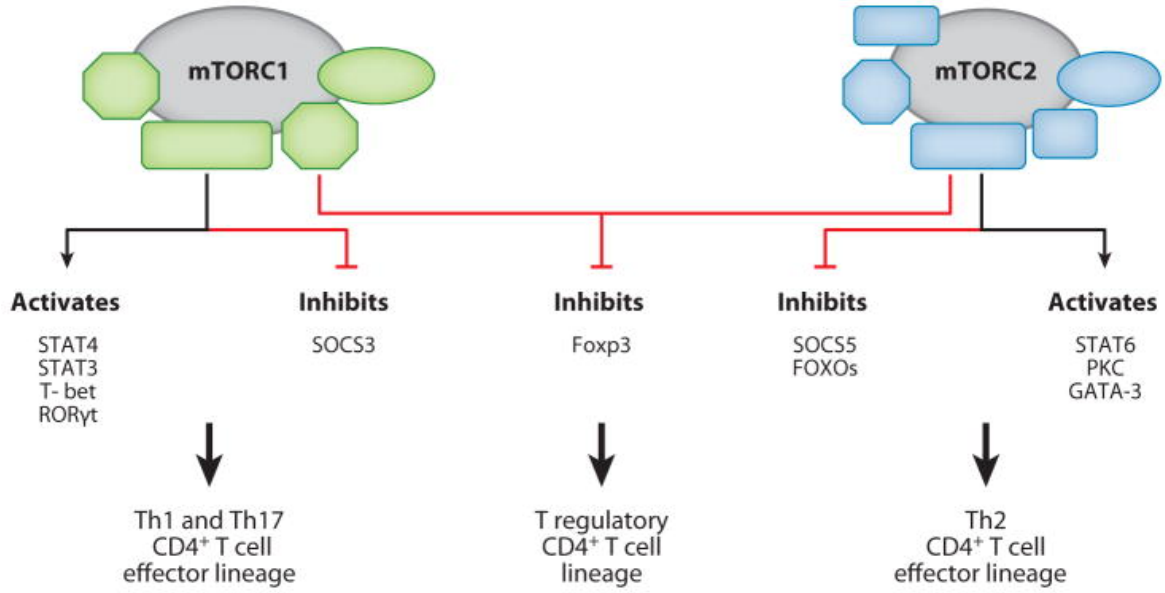

Fig. 2 mTOR in $CD4^+$ T cell differentiation. Both mTOR kinase complexes regulate of $Foxp3^+$ expression in $CD4^+$T cells, whereas Th1, Th2, and Th17 get either regulated by mTORC1 or mTORC2. mTOR complexes inhibit and/or activate different transcription factors that lead to the differentiation of the different $CD4^+$ T cell phenotypes. Studies indicate that T cells deficient in mTORC1 do not follow the regulatory T cell pathway. However, both mTOR complexes need to be deficient or inhibited to drive the generation of Tregs; suggesting that mTORC1 and mTORC2 regulate $CD4^+$ $CD25^+$ $Foxp3^+$ generation. (Delgoffe *et al.*, 2009) The inhibition of both complexes lead to a profoundly increased TGFb sensitivity in culture. Therefore, increased TGFb concentrations promote Treg generation. In fact, Treg generation with rapamycin treated T cells is also TGFb dependent. (Delgoffe *et al.*, 2009). Figure from (Powell *et al.*, 2012)

Compared to control DCs, rapamycin stimulated DCs have an increased capacity to stimulate the expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ cells, but impaired T effector cell (Teff) expansion. (Turnquist *et al.*, 2007)

Initially, it was suggested that rapamycin inhibits proliferation of T effector cells and/or promotes their death. Another possibility was that it promoted iTreg cell induction / expansion or a combination of effects on Teff and Treg cells. (Bilate and Lafaille, 2012)

1.2.3.3 mTOR pathway

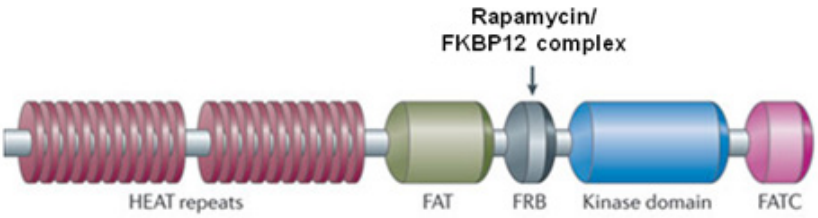

Fig. 3 Primary structure of mTOR with rapamycin/FKBP12 complex. mTOR is a conserved 289-kDa serine/threonine protein kinase. It is composes of two consecutive N-terminal HEAT (huntingtin, elongation factor 3, PP2A and TOR) domains, which mediate protein-protein interactions, followed by a FRAP, ATM, and TRRAP (FAT) domain. Rapamycin binds as a complex with FRBB12 to the FRB domain and thereby inhibits the activity of the kinase. The carboxy FAT (FATC) domain maintains the structural integrity of the protein. (Hay and Sonenberg, 2004; Powell *et al.*, 2012). Figure edited from (Benjamin *et al.*, 2011)

The mammalian target of rapamycin (mTOR, also known as FRAP) is a serine/threonine protein kinase within the family of phosphatidylinositol-3 kinase-related kinases (PIKKs) and encoded by the MTOR gene. (Brown *et al.*, 1994; Lovejoy and Cortez, 2009; Marone *et al.*, 2008) mTOR forms two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). (Araki *et al.*, 2010)

Rapamycin forms a complex with the intracellular cytosolic protein FKBP12 that binds to a C terminal domain of mTOR, termed FRB (FKB12-rapamycin binding). (Brown *et al.*, 1994; Hay and Sonenberg, 2004) The FKPB-12-rapamycin complex specifically binds to mTORC1 and inhibits its activity and a subset of mTOR functions. Prolonged treatment with rapamycin also results in a decreased activity of mTORC2 in some cells. (Araki *et al.*, 2010) The bound FKBP-12-rapamycin complex inhibits cell cycle progression from G1 to the S phase in T cells. (Magnuson *et al.*, 2012)

mTOR signaling is often upregulated in cancer. mTOR inhibitors demonstrated in preclinical trials that they might be effective against lung carcinoma. (Zarogoulidis *et al.*, 2014) Rapamycin was also the first drug that was able to show an extended maximal lifespan in both genders in mouse what might be due to prevent/postpone death from cancer. (Harrison *et al.*, 2009)

1.2.3.4 Rapamycin treated DCs

In immature $CD11c^{+}$ MHC^{lo} DCs it was observed that treatment with rapamycin leads to impaired macropinocytosis and mannose receptor-mediated endocytosis, which are the two major pathways for antigen uptake. (Hackstein *et al.*, 2002) Flt3L DC expansion is mTOR dependent and therefore treatment with rapamycin impairs Flt3L induced DC maturation *in vitro*, whereby pDCs and $CD8^{+}$cDCs were most affected. (Sathaliyawala *et al.*, 2010) However, impaired maturation through treatment with rapamycin does not

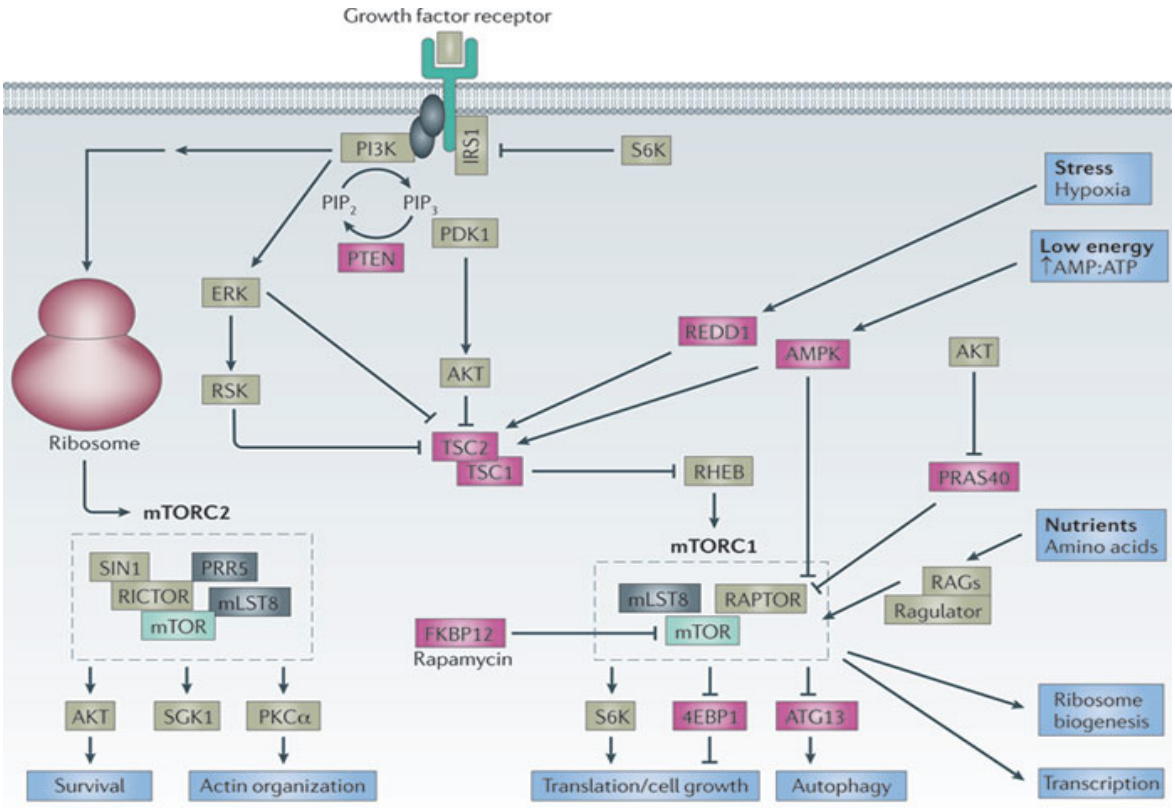

Fig. 4 mTOR signaling network and output of mTOR complex 1 (mTORC1) and mTORC2. Rapamycin binds intracellular to FKBP12 and forms a complex that binds directly to mTORC1. This inhibits mTORC1 activity, but is also decreasing to a lesser degree mTORC2 activity. Output of mTORC1 in regard to translation/cell growth or autophagy is dependent on factors such as nutrients, AMP-ATP energy levels or stress. mTORC1has a role in translation and transcription, lipid synthesis, nutrient influx and cellular energy levels as well as autophagy. mTORC2 coordinates cytoskeletal actin organization and is a significant factor in development and metastasis of cancer. For a more detailed description of all factors included in this figure see (Benjamin *et al.*, 2011)

impair their function to expand $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, but the expansion of Teff cells. (Turnquist *et al.*, 2007)

In a more recent study, it was shown that MyD88 signaling is required for Th17 development and proliferation by activating the mTOR signaling pathway. In particular, T cells deficient in MyD88 do not have a global mTOR defect, but are not able to integrate the IL-1 and IL-23 signal and sustain mTOR activity which is essential for terminal differentiation / maintenance of Th17 cells. IL-1 and IL-23 is mainly produced by APCs. It is suggested by the authors that the inability to integrate these signals specific for Th17 may not affect Th1 differentiation, although mTORC1 is involved in both responses. (Chang *et al.*, 2013)

1.2 Clinical perspective on Tregs

$Foxp3^+$ $CD25^+$ $CD4^+$ T cells are capable of inhibiting self-reactive T cells and therefore prevent autoimmune diseases and maintaining immunological tolerance. A more complete understanding of the processes involved in establish or maintaining tolerance has implications on the treatment and/or prevention of autoimmune disorders, allergies, organ transplant rejection, and immune responses to cancer. (Zwar *et al.*, 2006) The loss of self tolerance leads to autoimmune diseases such as systemic lupus erythematosus, type I diabetes or allergic asthma where also pDCs may play a critical role in the generation of $Fop3^+$ Tregs. (Kaneta *et al.*, 1986; Bluestone *et al.*, 2010; Choi *et al.*, 2012; Maazi *et al.*, 2013)

1.2.4 Organ transplantation

The recognition of non-self-antigens typically leads to rejection of an allograft. The ultimate goal of solid organ transplantation is to achieve immunological tolerance by eliminating alloreactive lymphocytes in the absence of immunosuppressive medication. (Keller and Burlingham, 2011) One of the current used immunosuppressive drug in allograft rejection is rapamycin and its analogues, though it is not fully understood how rapamycin specifically acts on different cell types. (Fantus and Thomson, 2015)
All thymocytes undergo positive and negative selection in the thymus. The thymus is considered the central organ of self versus non-self discrimination. T cells play a critical role and immune tolerance is achieved by two different processes. (Griesemer *et al.*, 2010)

First, by deletion of self-reactive T cells (T cells with TCRs that have high affinity to self-antigens) in the thymus before these cells reach maturity. This process is referred to as central tolerance. Moreover, it was demonstrated that efficient antigen presentation by DCs is important for negative selection and essential to induce complete immunological tolerance. (Griesemer *et al.*, 2010)
Second, by suppression of self-reactive T cells in the periphery which have escaped thymic elimination. This process is referred to as peripheral tolerance. (Mueller, 2010)
In both mechanisms regulatory T cells are capable of depleting these autoreactive T cells. In particular, $CD4^+$ $CD25^+$ Tregs demonstrated that they are capable of inducing transplantation tolerance and maintain transplantation tolerance after it was acquired in skin grafts. (Graca *et al.*, 2002; Cobbold *et al.*, 2004). Mice which lack the ability to induce both $Foxp3^+$ cell types ($CD4^+$ and $CD8^+$ $Foxp3^+$ cells) die earlier as a result of graft-versus host disease (GVHD) associated with allogeneic stem cell transplantation. (Freiherr von Hornstein *et al.*, 1988)

1.2.5 Allergic Diseases

Allergic diseases are clinical manifestations of an atopic, allergen-specific immune response that is directed against harmless environmental allergens. A central tenet in this paradigm is the development of allergen-specific naïve $CD4^+$ T cells away from a tolerant or hypo-responsive mode towards a Th2-polarized effector pathway. Data from both humans and mice implicates Th2-polarized $CD4^+$ T cells as critical players in the allergic inflammatory cascade through the induction and maintenance of humoral and cellular/eosinophilic responses.

A common allergic disease is allergic asthma in which symptoms can be elicited by exogenous allergens such as house dust mite or pollen. (Lambrecht and Hammad, 2015) It was reported that Tregs maintain functional tolerance in murine asthma models by suppressing Th2 responses, mucous hypersecretion, airway hyperresponsiveness, and eosinophilia in the airways. (Larché, 2007) Th2 responses are the hallmark of allergic asthma and augment the inflammatory response by secreting the cytokines IL-4, IL-5, and IL-13. IL-4, as a central mediator of airway inflammation, drives the development of IgE antibodies against ovalbumin and leads to eosinophilia in BAL fluid. (Brusselle *et al.*, 1994) Mice lacking Foxp3 develop strong Th2 responses in the lungs and other mucosal tissues. (Josefowicz *et al.*, 2012)

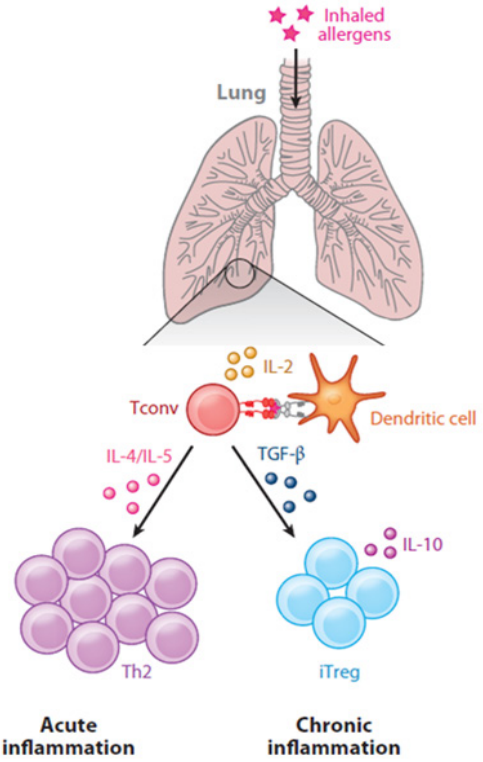

Fig. 5 Allergic asthma caused by inhaled allergens. DCs present the sampled antigen to a conventional T cell, depending on the cytokine, the DC promotes the conversion of the conventional T cell to a induced Treg or Th2 cell. In contrast to Th2 cell response, iTregs limit the immune response to allergens and control Th2 damage. Figure edited from {Bilate 2012 #62}

Mice that are exposed to an aerosolized antigen such ovalbumin (in the absence of adjuvants) usually develop airway tolerance mediated by $Foxp3^+$ $CD4^+$ T cells. Therefore, $Fopx3^+$ $CD4^+$ cells mediate the inhibition of allergic airway inflammation in mouse models. (Ostroukhova *et al.*, 2004) The suppression of the inflammatory response through $CD4^+$ $CD25^+$ $Foxp3^+$ T cells is IL-10 and TGFb dependent. (Kearley *et al.*, 2005)

2 Methods

2.1 Material

AIM-V: Invitrogen 087-0112DK

MACS buffer: 0.5% Bovine Serum Albumin (BSA), 2mM EDTA in 1xPBS

FACS buffer: 10% FBS (Gemini, heat inactivated), 2mM EDTA in 1x PBS

Human serum (HS):Heat inactivated in a water bath for 30 min at 56°C and filtered.

Dendritic Cell Media (DCM): 0.1% 2-ME, 10% FBS (Gemini, heat inactivated), 1% Glutamine (100x), Penicillin (100 µg/ml) + Streptomycin (100 µg/ml) in Iscove's DMEM (1x) (Corning cellgro)

T Cell Media (TCM, TIMM): 0.05% 2-ME, 10% FBS (Gemini, heat inactivated), 1% Glutamine (100x), 15 mM HEPES buffer, 1.5 mM Sodium Pyruvate in RPMI-1640 medium (1x) (all Corning cellgro). TIMM media contains the same ingredients, except the RPMI-1640 (1x) medium does not contain phenol red in TIMM media.

Ovalbumin (OVA):Grade V (Sigma Aldrich Cat#:A5503)

Lipopolysaccharide (LPS): Sigma L2654-1MG

Rapamycin: Sigma R8781

Interleukin 2 (IL-2): Roche

Interleukin 4 (IL-4): Recombinant Human IL-4 (carrier-free) (BioLegend, Lot#B154573)

Collagenase D: Liberase ^{TM} Research Grade from Roche

GM-CSF: Recmobinant Human GM-CSF (carrier-fee) (BioLegend, Lot#B152921)

TGFb1 (human): Recombinant Human TGFb1 (R&D Systems Cat.#240-B)

2.2 Murine protocols

2.2.1 Dendritic cell protocols

2.2.1.1 CD11c$^+$ DC isolation

Dendritic cells were purified from murine spleens harboring Flt3L expressing tumors (sacrificed latest 14 days after injection with tumor cells). Flt3L increases the amount of CD11c$^+$ DCs from 2% in a wild type mouse to about 40% in spleen and is essential to generate a sufficient amount of DCs for subset isolation for *in vitro* studies and *in vivo* adoptive transfer of DC subsets. (Miller *et al.*, 2003)

Spleens are removed immediately after sacrificing the mouse and RPMI which contains liberase was injected in each spleen to obtain a higher yield of DCs by enzymatic disaggregation. Spleens are chopped into pieces, pooled, and incubated for 30min at 37°C. With the back of a syringe plunger the tissue fragmenst are forced through a cell strainer into a 50 ml tube to generate a single cell suspension in MACS buffer and centrifuged at 350g for 5min. The supernatant is discarded and the pellet resuspended in RBC lysis buffer (5 ml/spleen) for 1 min. Then tube is filled up with MACS buffer to stop the lysis reaction and centrifuged at 350g for 5 min. The supernatant is discarded and total cells are counted with Turk solution (Ricca Chemical Company).

Unlike in humans, CD11c is expressed in mice on all defined subsets. FcR blocking reagent for mouse (1:200) is added and incubated for 5 min on ice. The single cell suspension obtained from spleen (250x10^6/ml) is positively selected by anti-CD11c-coated magnetic beads (Miltenyi Biotec) and separated on a magnetic column according to manufacturer specifications. A 2-step approach was used by using a second column to yield a more pure population. (Miltenyi Biotec, 2009) The purity of the positive CD11c enriched fraction (total DCs (= panDCs)) was verified by staining of an aliquot with anti-CD11c (Pe-Cy7) (1:200) for 20 min on ice. CD11c$^+$ cell purity was over 98% in all experiments.

2.2.1.2 DC subset isolation

Cells were stained with the following antibodies (1:200): CD11c (Pe-Cy7), CD4 (Pacific Blue), CD8a (APC), CD11b (FITC), PDCA-1 (PE).

Five subsets were sorted by flow cytometry into DCM as followed: CD4 (CD11c$^+$ CD4$^+$ CD8a$^-$ CD11b$^+$), CD8a (CD11c$^+$ CD4$^-$ CD8a$^+$ CD11b$^-$), CD11b (CD11c$^+$ CD4$^-$ CD8a$^-$ CD11b$^+$),triple negative subset (CD11c$^+$ CD4$^-$ CD8a$^-$ CD11b$^-$), pDC (includes CD11c^{int} PDCA-1$^+$ CD11b$^-$ CD4$^-$ (65%) and CD11c^{int} PDCA-1$^+$ CD11b$^-$ CD4$^+$ (35%) pDC subsets).

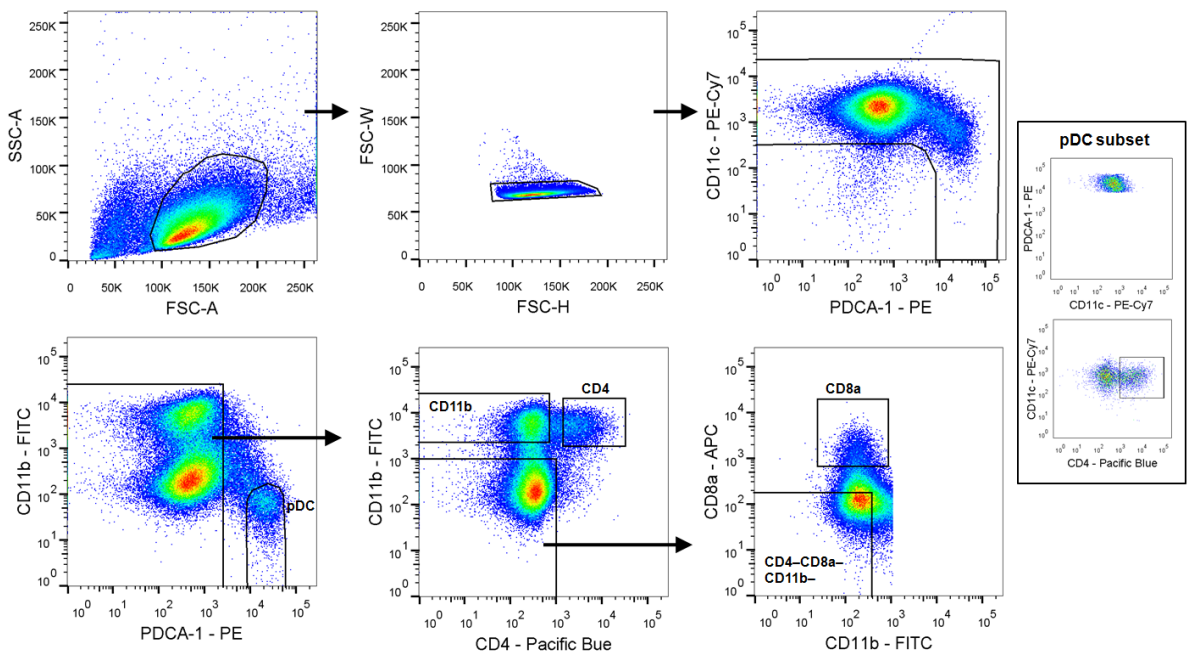

Fig. 6 FACS sorting strategy for murine DC subsets from pre-purified CD11c^{+} cells from spleen. Additionally, pDCs are depicted on the right. pDCs are a pure population of CD11c^{int} and PDCA-1^{hi} cells, but pDCs contain two subpopulations. The gated pDC population (CD11c^{int} PDCA-1^{hi} CD4^{+}) consist of 36% of the total pDC population.

After sorting, cells were centrifuged at 350g for 5 min, resuspended in DCM, and percentage of living cells counted with trypan blue.

2.2.1.3 Macrophage and lung DC isolation

10 female C57BL/6 mice were exasanguinated and perfused by injecting cold 1x PBS in the right ventricle (20 ml) to exclude nontissue-resident cells (Fig. 8). A incision was made into the trachea and bronchoalveolar cells were removed through bronchoalveolar lavage (BAL) by injecting and removing 1 ml 1x PBS two times with an appropriately sized sterile needle. Lung lobes were taken from right and left lung. Choped lung lobes were digested in RMPI 1640 1x (Gibco) with Collagenase D (3mg/ml) and DNase (50 µg/ml) for 1h at 37°C. With the back of a syringe plunger the lungs were forced through a cell strainer in a 50 ml tube to a single cell suspension in MACS buffer and centrifuged at 350g for 5min. After incubation with mouse Fc blocking antibody (2.4G2), cells were stained with I-Ab/AF/AE (Pacific Blue), Siglec F (PE), CD11c (Pe-Cy7), CD45 (APC). Lung resident DCs (CD11c^{+} CD45^{+} Siglec-F^{lo} MHC-II^{lo}) and tissue resident alveolar macrophages (CD11c^{+} CD45^{+} Siglec-F^{+} MHC-II^{hi}) were sorted from lung tissue. Alveolar macrophages were also sorted from BAL fluid. (Soroosh *et al.*, 2013; Segura and Amigorena, 2014)

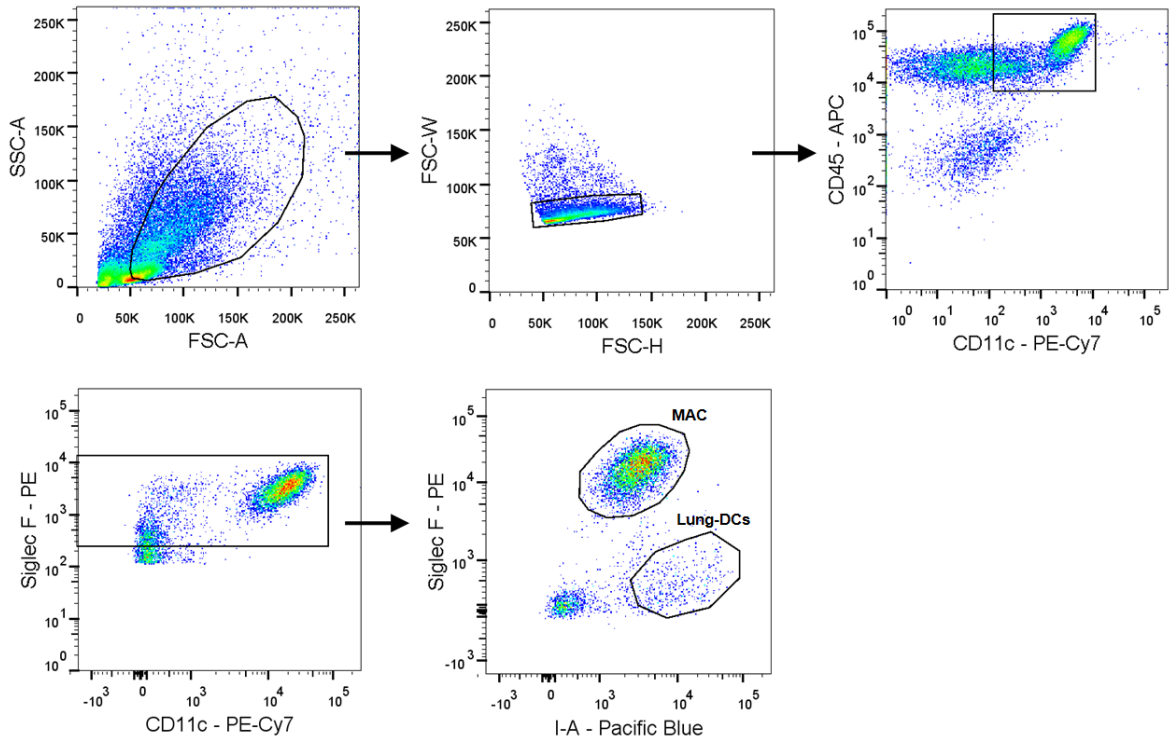

Fig. 7 FACS sorting strategy for macrophages and lung DCs from perfused lung tissue and for broncho-alveolar macrophages from BAL fluid.

2.2.1.4 DC pulsing with ovalbumin

panDCs and DC subsets were pulsed with 100 µg/ml sterile chicken ovalbumin prepared in three conditions; treated with 100 ng/ml rapamycin + TGFb (20 ng/ml), LPS (1µg/ml), or untreated (control group) for 2h at 37°C in DCM at a concentration of 1x10^6 /ml for DC subsets and 5x10^6/ml for panDCs. In one experiment the optimal ovalbumin concentration for murine DC pulsing was empirically determined with 100 µg/ml from concentrations of 1, 10, 100, and 500 µg/ml (data not shown). After 2h pulsing, all samples were extensively washed (4x) with DCM (2x) followed by TCM / TIMM (2x) and centrifugated after every single washing step at 350g for 5 min to remove LPS and rapamycin. Cells were resuspended in TCM for *in vitro* culture or TIMM for video-microscopy.

2.2.2 T cell isolation protocol

2.2.2.1 Naïve T cell isolation from OT-II Foxp3-GFP mice

T cells were purified from lymph nodes (cervical, paraaortic, poplitea, inguinal, brachial, axillary) and spleen from transgenic OT-II Foxp-3-GFP Rag$^{het/KO}$ mice. OT-II Foxp3-GFP mice are transgenic for the T-cell receptor recognizing OVA peptide 323-339 and carry an endogenous GFP expressing Foxp3 reporter that is used to detect and visualize Tregs.

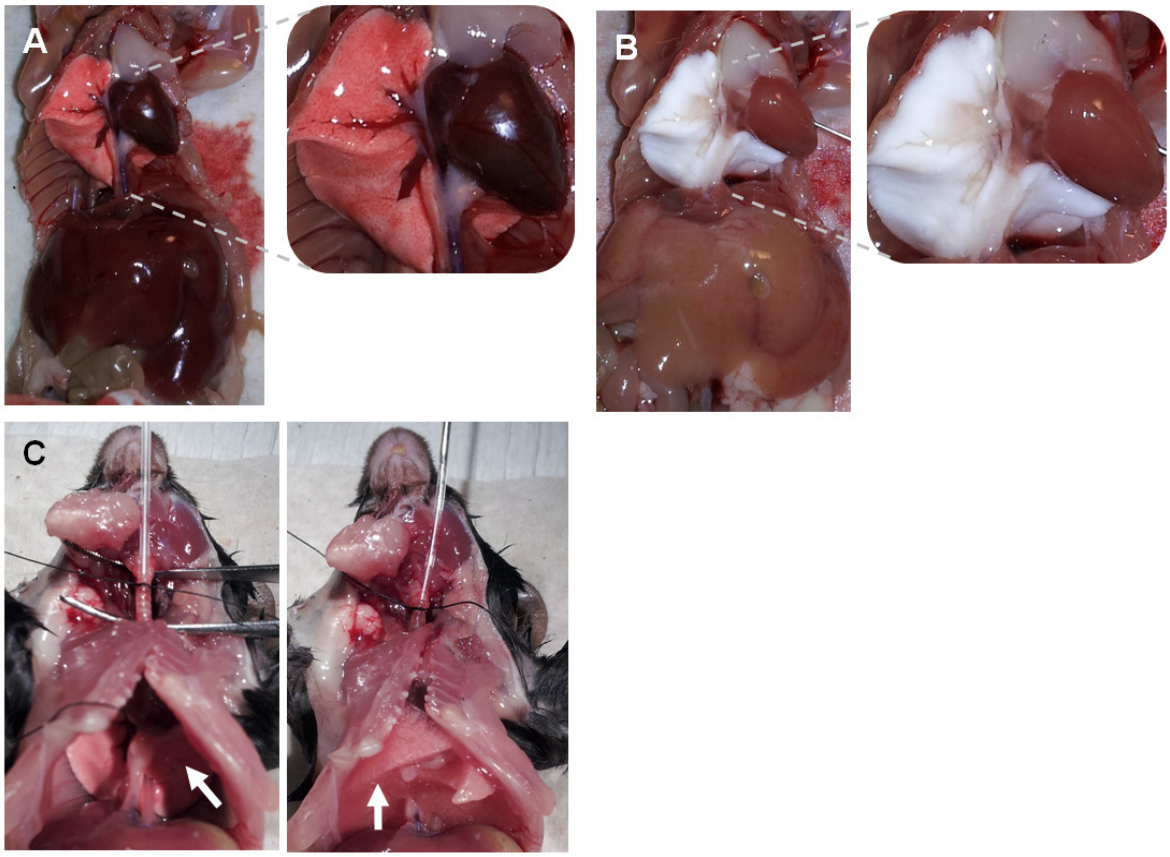

Fig. 8 Isolation of macrophages and lung-DCs. **(A)** the chest and abdomen of the mouse are opened to access the right ventricle. The inferior vena cava is cut and cold PBS is injected into the right ventricle. The magnification depicts the right lung and the heart. **(B)** After perfusion, the lung is visibly bright white as well as the branches of the pulmonary artery are blood-free, but also other organs (liver, heart) are visibly brighter due to washing out the blood from these tissues. **(C)** A incision is made in the trachea and a tube is inserted and fixed with a string for bronchoalveolar lavage (BAL). The left arrow points to the physiological collapsed lung, whereas the arrow on the right indicates the over-inflated lung due to the injection of PBS for BAL.

Spleen and LNs are processed seperately by forcing the tissues through a cell strainer to obtain a single cell suspension in MACS puffer and centrifuged at 350g for 5min. The pellet of the spleen suspension was lysed for 1 min with 1 ml RBS lysis puffer and filled up with MACS puffer to stop the reaction. The LNs and lysed spleen samples were combined and total cell numbers counted with Turk solution.

Cell suspensions from OT-II Foxp3-GFP Rag^{het} mice were enriched with the $CD4^+$ $CD62L^+$ T cell Isolation Kit II mouse (Miltenyi Biotec) to obtain an enriched fraction of naïve T cells, whereas OT-II Foxp3-GFP Rag^{KO} cell suspensions were directly stained and sorted by flow cytometry.

Total cells were counted with Turks solution to obtain total cell numbers and resuspended at a concentration of 250x10^6 cells/ml in MACS buffer. 100 µl of $CD4^+$ T cell Biotin-Antibody Cocktail II (biotin-conjugated monoclonal antibodies against CD8a, CD45R, CD11b, CD25, CD49b, TCRγ/δ, and Ter-119) for each 100 million cells were added and

incubated for 10 min at 4°C. 300 µl of buffer and 200 µl of Anti-Biotin Microbeads per 100 mio cells were added and incubated for 15 min at 4°C. Cells were washed and resuspended in MACS puffer at a concentration of 200 mio cells/ml for magnetic separation. Non-$CD4^+$ cells were depleted by magnetic separation according to the manufacturer's manual.

Finally, cells were stained 1:200 on ice for 30min with CD25 (PE), CD69 (PE), CD4 (Pacific Blue), CD44 (Pe-Cy7), and CD62L (APC). Naïve $CD4^+$ $CD25^-$ $CD69^-$ $CD44^-$ $CD62L^+$ $Foxp3^-$ T cells were sorted in TCM and used for *in vitro* culture or *in vivo* experiments (adoptive transfer).

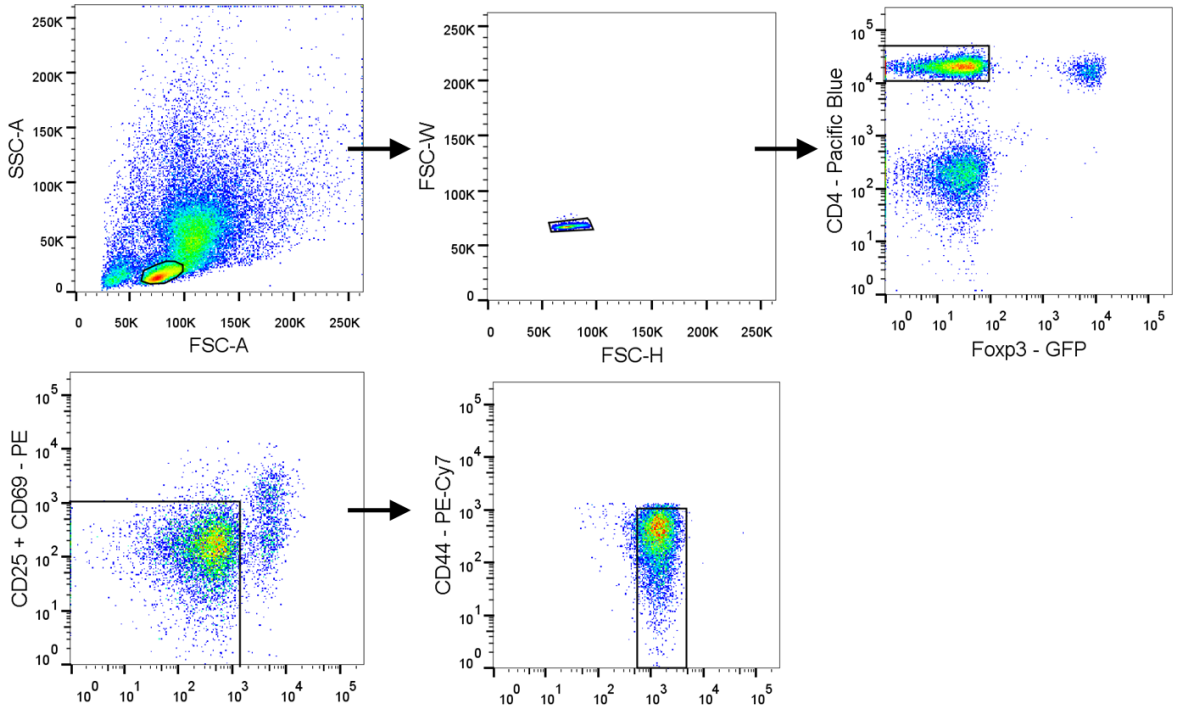

Fig. 9 Naïve T cells isolation from OT-II Foxp3-GFP mice. Sorted cells are naïve $CD4^+$ $CD25^-$ $CD69^-$ $CD44^-$ $CD62L^+$ $Foxp3^-$ T cells

2.2.3 DC / T cell co-cultures

2.2.3.1 Murine DCs and naïve T cells cocultivation

Genders were always matched from DC donor and T cell donor mice. DCs were cocultured with FACS purified naïve $CD4^+$ $CD25^-$ $CD69^-$ $CD44^-$ $CD62L^+$ $Foxp3^-$ T cells in a ratio of 10:1 and/or 5:1 with total cell numbers of 1x10^5 or 2x10^5 T cells in each 96 well plate. T cells were cocultured with DCs in four conditions: control DCs + T cells, LPS treated DCs + T cells, rapamycin and TGFb treated DCs + T cells, and control DCs with IL-2 (20 U/ml) and TGFb (20 ng/ml) + T cells (positive control). Cells were split at day 3.5 and fresh media with IL-2 (20 U/ml) was added. Cultures were harvested and OT-II Foxp3-GFP antigen-specific activation was measured at day 5.5 .

2.2.3.2 cocultivation of macrophages and lung DCs

A female OT-II Rag^{KO} Foxp3-GFP mouse was used as T cell donor and naïve $CD4^+$ $CD25^-$ $CD69^-$ $CD44^-$ $CD62L^+$ $Foxp3^-$ T cells were cocultured with macrophages and DCs at a ratio of 10:1 (total 125x10^5 cells). T cells were prepared as described in section "Naïve T cell isolation from OT-II Foxp3-GFP mice". Macrophages and lung DCs were pulsed with 100 µg/ml sterile ovalbumin at a cellular concentration of 1x10^6/ml for 2h at 37°C.

As a control, we isolated DCs from mice with Flt3L expressing tumors (see "Dendritic cell isolation from mouse") from spleen and treated the total DC population (panDCs) with 100 ng/ml rapamycin + TGFb (20 ng/ml), LPS (1µg/ml), or did not treat the DCs with any agent (control group) in DCM at a cellular concentration of 5x10^6/ml for 2h at 37°C. This control experiment was used to directly compare the potency of splenic it-DCs with macrophages and lung tissue resident DCs.

T cells were cocultured with DCs in four conditions: control DCs + T-cells, LPS treated DCs + T-cells, rapamycin and TGFb treated DCs (itDCs) + T cells, and control DCs with IL-2 (20 U/ml) and TGFb (20 ng/ml)+ T cells (positive control). After 2 hours of pulsing, all samples were extensively washed (4x) with DCM (2x) followed by TCM (2x). Cells were resuspended in TCM for *in vitro* culture. Cells were not split and no IL-2 was added. Cultures were harvested and OT-II Foxp3-GFP antigen-specific activation was measured at 5.5 .

2.2.3.3 Staining of murine DC and T cell co-cultures

Cultures were harvested after day 5. Staining was performed with CD4 (Brilliant Violet 421), CD25 (PE), viability dye 506, and intracellular Foxp3 (Alexa 647) after fixation of the

cells. In preliminary experiments, cells were additionally stained with GARP, FR4, CD127, CD39, and GITR. The analysis of preliminary data suggested that the staining with CD25, CD4, and Foxp3 are sufficient for gating of our desired Treg population. The endogenous Foxp3-GFP signal was comparable to the Foxp3-APC signal (<±5% difference) gained by intracellular staining.

2.2.4 Murine *in vivo* protocols

2.2.4.1 DC mediated Foxp3$^+$ Treg induction in vivo

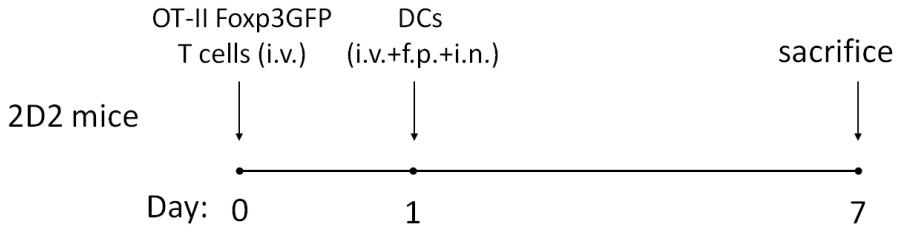

Fig. 10 Schematic of the approach to induce Foxp3$^+$ CD25$^+$ Tregs from adoptively transferred naïve CD4$^+$ T cells in 2D2 mice. OT-II cells are injected intravenous (i.v.) at day 0. 24 hours later, DCs are adoptively transferred intravenous (i.v.), in the foot pad (f.p.), and intra nasal (i.n.). Mice are sacrificed at day 7.

Naïve CD4$^+$ CD25$^-$ CD69$^-$ CD44$^-$ CD62L$^+$ T cells were FACS purified from a male CD45.1.2 OT-II Rag^{KO} Foxp3-GFP mouse. CD11c$^+$ DCs were isolated from a βact-RFP mouse with a kit from Miltonyi Biotec (see "Dendritic cell isolation protocols"). DCs were pulsed with 100 µg ovalbumin and prepared in the following conditions: untreated, LPS treated, and rapamycin + TGFb treated (concentrations, see "DC pulsing with ovalbumin"). Recipients were male CD45.2 2D2 TCR transgenic mice (n=3). 2x10^5 naïve CD45.1.2 CD4$^+$ OT-II Foxp3-GFP T cells were adoptively transferred i.v. (retro-orbital) in each mouse. 24h later 1x10^6 DCs were adoptively transferred i.v. (retro-orbital) and 7.5x10^5DCs each into the left foot pad (f.p.) and intra nasal (i.n.). Mice were sacrificed at day 7. Spleen, peripheral blood, cervical and tracheal/bronchial lymph nodes (LNs) were harvested. Popliteal and inguinal LNs were also taken from both sides, but processed separately to detect a difference between the side of DC injection (left foot pad) and the non-draining lymph nodes on the right side.

5 million cells of the draining LNs, non-draining LNs (right), spleen, peripheral blood, and cervical + tracheal/bronchial LNs were stained. Staining was performed with CD45.1 (PE-Cy7), CD45.2 (Pacific Blue), CD25 (PE), CD4 (PerCP-Cy5.5) for 1h 1:200 on ice. Cells were fixed for 15min at +4°C and prepared for intracellular staining with the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BioLegend) according to the protocol. (Biolegend, 2005) Samples were stained for 12h with Foxp3 (Alexa 647) 1:400 at +4°C in FACS buffer for Foxp3 detection.

2.2.4.2 *it-DC therapy in OVA-based asthma models*

Fig. 11 Sensitization and challenge model of allergic asthma. Mice are sensitized at day 0 and day 14 with ovalbumin (OVA;10 µg) and aluminium hydroxide ($AlOH_3$; 1 mg). it-DC subsets CD8a and triple negative, as well as panDCswere administered intra nasal (i.n.) at day 16. The control group (n=5) did not receive OVA. OVA challenging starts at day 17 up to day 20 and was inhaled as an 1% aerosol. All mice were sacrificed at day 21.

The aim of this experiment is to induce an allergic asthma-like reaction in the airways and hamper the allergic response (eosinophilia) by administrating it-DCs. Systemic intraperitoneally (i.p.) immunization of ovalbumin with the adjuvant aluminium hydroxide ($AlOH_3$) leads to an augmented Th2 response after repeated administration (day 0 and day 14). Systemic sensitization is necessary, because airway tolerance can develop if OVA is solely administered by inhalation. (Kumar *et al.*, 2008; Nials and Uddin, 2008)

C57BL/6 mice were sensitized by injection of 500 µl sterile filtered OVA in 1x PBS (20 µg/ml) and $AlOH_3$ (2 mg/ml) i.p. on day 0 and day 14. DC subsets were sorted from spleen of Flt3L (day12). $CD11c^+$ DCs (panDCs) were isolated by magnetic labelling with over 98% purity (Miltonyi Biotec). DC subsets $CD8a^+$ ($CD8a^+$ $CD11c^+$ $CD4^-$ $CD11b^-$) and $CD11c^+$ $CD8a^-$ $CD4^-$ $CD11b^-$ (triple negative subset) were purified by FACS. $CD8a^+$ DCs, triple negative DCs, and panDCs were pulsed with OVA (100 µg/ml) and treated with 100 ng/ml rapamycin + TGFb (20 ng/ml) for 2h at 37°C at a concentration of 5x10^6 cells/ml. All DCs are it-DCs after rapamycin treatment.

Four groups (n=20) were used. At day 16, $CD8a^+$ DCs (n=5), triple negative DCs (n=5), panDCs (n=5), or no DCs (control group, n=5) were administered intra nasal (i.n.) at a concentration of 5x10^5 / mouse. OVA challenges were performed daily starting on days 17 thru 20 via nebulization of a 1% Ova solution in 1x PBS for 20 min each day. All mice were sacrificed on day 21. Lungs and LNs were harvested to evaluate the induced inflammation in the different groups.

2.3 Human protocols

2.3.1 DC protocols

2.3.1.1 CD11c^{+}DC and monocyte isolation from blood

200 ml of human blood from healthy donors were used for each experiment. 50ml Leucosep^{TM} (Greiner Bio-One) tubes with porous membranes were overlayed with Ficoll-Plaque (GE Healthcare, density 1.077 ± 0.001 g/ml) and centrifuged for 30 sec at 1000g. 25 ml of blood are each transferred into 50ml Leucosep^{TM} (greiner bio-one) tubes and spun for 15 min at 800g with deceleration switched off. The platelets layer was removed with a sterile glass pipette. The lymphocyte/PBMC layer (~10ml) was harvested and transferred into 50ml tubes. The enriched lymphocyte fraction is washed with 1x PBS and spun for 10 min at 250g. The supernatant was removed, RBC lysis buffer added, and incubated for 10 min at room temperature, followed by washing with 1x PBS. The suspension was filtered through a 70 µm strainer into 50 ml tubes and centrifuged 10min at 250g. The suspension was resuspended into MACS buffer and total cell numbers were counted with Turk blood diluting fluid (Ricca Chemical Company). (Greiner Bio-One, 2013)

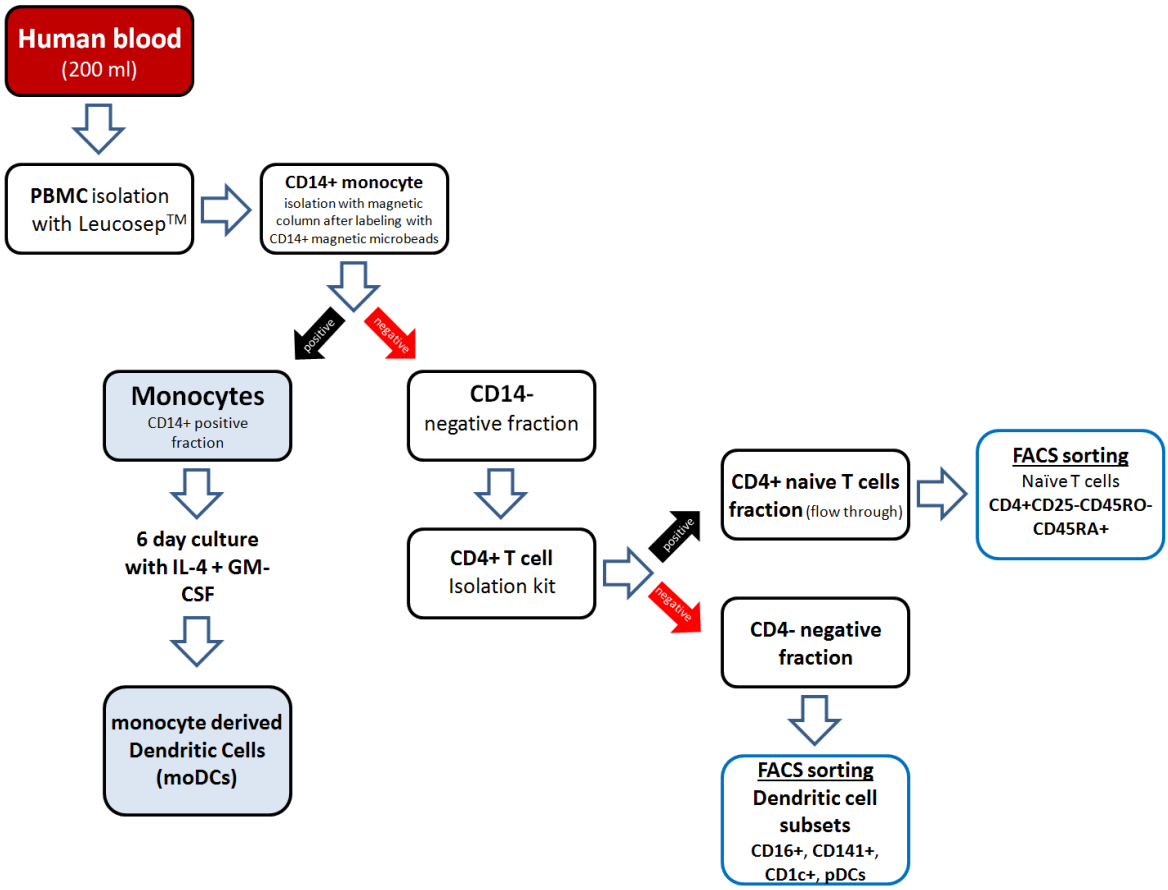

Fig. 12 Protocols for human blood DC subset isolation, monocyte isolation, naïve CD4^{+} T cell isolation, and cultivation of moDCs from human blood. All protocols are described in more detail in the following sections.

2.3.1.2 *CD14$^+$ monocyte-derived DC protocol*

The total lymphocyte population was obtained from blood. Monocytes were positively selected by anti-CD14-coated magnetic beads (Miltenyi Biotec) and separated on a magnetic column according to the manual. (Miltenyi Biotec, 2013a) To monitor the purity of the obtained CD14$^+$ monocyte fraction we routinely stained the CD14$^+$ enriched sample for flow cytometry.

Purified CD14$^+$ mononuclear cells were resuspended in different culture media (Aim-V, TCM, DCM, or AIM-V + 10% heat-inactivated human serum) and plated at 1x10^6/ml in 6-well plates in medium supplemented with GM-CSF (1000 U/ml) and IL-4 (500 U/ml). After 3 days of culture, additional fresh medium with GM-CSF and IL-4 in the same concentrations was added. On day 6, cells were harvested and treated with rapamycin (10 ng/ml) and TGFb (2ng/ml), LPS (1µg/ml) or left untreated (control group) for 18h at 37°C.

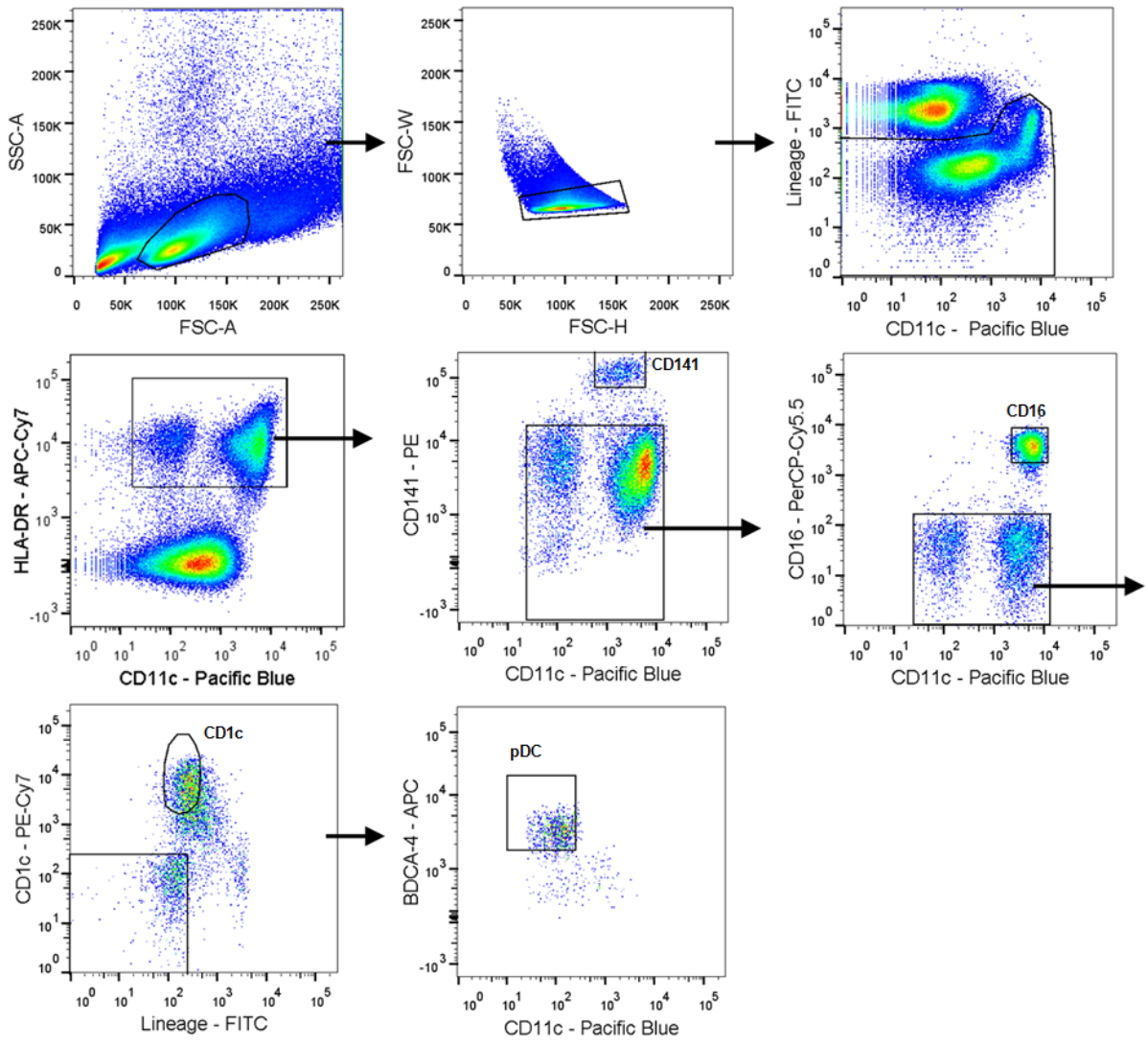

Fig. 13 FACS sorting strategy for human DC subsets from pre-purified cells from human blood. Lin- cells do not express CD3, CD19, Ter119, NK1.1, or B220 antigens.

2.3.1.3 Human DC subset isolation

The positive fraction from the human $CD4^+$ T cell isolation kit II was stained for Dendritic cell subset sorting (Fig. 13) with the following anti-human antibodies according to the manuals (all BioLegend). CD11c (Brilliant Violet 421), Lineage cocktail (CD3, CD14, CD19, CD20, CD56) (FITC), CD141 (PE), CD16 (PerCP-Cy5.5), CD1c (Pe-Cy7), BDCA-4 (APC), HLA-DR (APC-Cy7). All sorted subset were sorted as follows: CD141 ($CD11c^+$ $HLA\text{-}DR^+$ $Lineage^-$ $CD141^+$), CD16 ($CD11c^+$ $HLA\text{-}DR^+$ Lin^- $CD16^+$), CD1c ($CD11c^+$ $HLA\text{-}DR^+$ Lin^- $CD1c^+$), pDC ($CD11c^-$ $HLA\text{-}DR^+$ Lin^- $BDCA4^+$).

All DCs were prepared in three conditions; treated with rapamycin (10 ng/ml) + TGFb (2 ng/ml), LPS (1µg/ml), or untreated (control group) for 2h at 37°C in AIM-V or AIM-V medium with 10% heat inactivated human serum from the same donor.

2.3.2 T cell isolation protocol

2.3.2.1 Naïve $CD4^+$ T cell isolation

The CD14 negative fraction (flow through / not magnetic labeled cells) was further used to enrich for naïve $CD4^+$ T cells with the human $CD4^+$ T cell isolation kit II (Miltenyi Biotec). We followed the protocol (Miltenyi Biotec, 2013b) and prepared the enriched cell fraction (negative fraction) for additional cell sorting by staining with the following fluorochrome conjugated antibodies: CD4 (APC-Cy7), CD25 (PE), CD45RO (Alexa 488), CD45RA (Brilliant violett 421) for 30min on ice. Prior to staining for cell sorting, the $CD4^+$ enriched fraction was stained with CD3, CD4, and CD56, and CD16 to ensure proper enrichment with the $CD4^+$ T cell kit.

Naïve $CD4^+$ $CD25^-$ $CD45RO^-$ $CD45RA^+$ T cells were sorted and used for *in vitro* culture with moDCs and blood isolated DCs. A fraction of the isolated naïve T cells was frozen at -80°C in 90% FBS and 10% DMSO for moDCs cocultivation that arise after 6 days in culture.

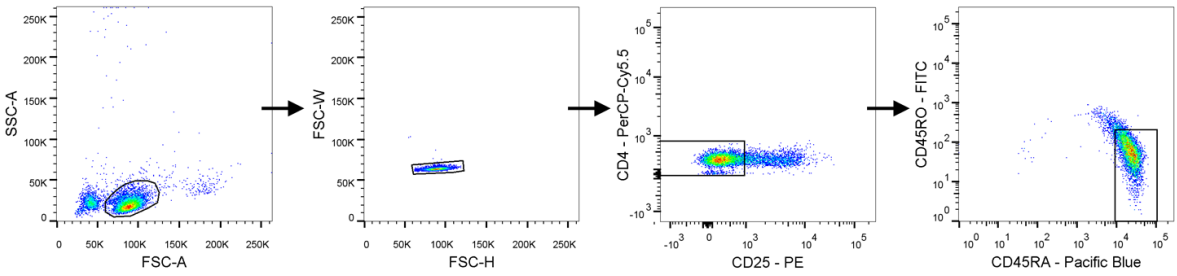

Fig. 14 FACS sorting strategy for human naïve $CD4^+$ $CD25^-$ $CD45RO^-$ $CD45RA^+$ T cells from a pre-enriched fraction of human blood.

2.3.3 DC/T cell co-cultures

2.3.3.1 DCs and naïve T cells cocultivation

DCs and moDCs were cocultured with naïve $CD4^+$ $CD25^-$ $CD45RO^-$ $CD45RA^+$ T cells in a ratio of 10:1 and/or 5:1 with a total cell number of 1x10^5 or 2x10^5 T cells in each 96 well plate. T cells were cocultured with DCs and supplemented with CD3 (10 ng/ml) in four conditions: control DCs + T cells, rapamycin and TGFb treated DCs + T cells, and control DCs + IL-2 (200 U/ml) and TGFb (2 ng/ml) as positive control. In some experiments, cells were splited at day3 and fresh media with IL-2 (200 U/ml) was added. Cells were cultured for 5.5 days. Naïve T cells were frozen at -80°C at the day of isolation from human blood and thawed as soon as moDCs arose at day 6 for cocultivation. Cells were harvested and washed with FACS buffer to prepare the cells for fluorochrome staining.

2.3.3.2 Staining of DC/T cell cocultures

Cells were stained with the following fluorochrome conjugated antibodies from BioLegend: CD45RA (Brilliant Violet 421), CD127 (Pe-Cy7), CD4 (APC-eFluor 780), CD25 (PE) in FACS buffer. The cells were stained in concentrations recommended by the manufacturer and incubated for 30 min on ice. The reaction was stopped with 1x PBS and centrifuged at 350g for 5 min. Cells were resuspended in 100 µl viability dye 506 (1:400) and incubated for 30 min at 4°C. The reaction was stopped with FACS buffer. Cells were fixed with the Foxp3 Fix/Perm Buffer Set (BioLegend) for intracellular staining of Foxp3 according to the provided manual. (Biolegend, 2012) Cells were intracellular stained with anti-human Foxp3 (eFluor 660) (5µl/sample in 100 µl total volumen) for 30 min at room temperature. After washing with 1x Perm buffer, samples were analyzed by FACS.

Prior to all stains, human cells were incubated on ice for 5 min with the Fc receptor blocking solution Human TruStain FcX (Biolegend).

2.4 Live-cell imaging

DCs (murine panDCs, murine and human DC subsets) and naïve T cells preparation followed the same protocols as for *in vitro* cultures (see above). Images were recorded with Vivaview FL Incubator Fluorescence Microscope (Olympus) for up to 5 consecutive days. This incubator provides optimal culture conditions and allows continous imaging of cells. Glass bottom dishes with 35mm diameter with 4-well silicon inserts from Ibidi (culture treated) and Mattek (Collagen coated) were used. T cells : DCs were plated in a ratio of 1:5 with a maximum of 50.000 T cells and 10.000 DCs per well.

Videomicroscopy in mouse

Murine RFP positive panDCs (red fluorescence in images and videos) and subsets were isolated from βact-RFP positive mice with Flt3L expressing tumors from spleen (see "Dendritic cell isolation protocols"). DCs used for imaging were treated with rapamycin (100 ng/ml) + TGFb (20 ng/ml) (it-DC group). Untreated DCs (control DCs), LPS (1µg/ml) treated, and a positive control (control DCs with TGFb (20ng/ml) + IL-2 (20 U/ml)) were cultured parallel as quality control.

Videomicroscopy in human

Human blood isolated DCs ($CD141^+$, $CD1c^+$, $CD16^+$, pDCs) were cultivated in TIMM medium in which FBS was replaced by 10% heat inactivated human serum from the same blood donor. Human samples were not stained, but analyzed for morphological characteristics and changes over time in culture.

3 Results

The ability to generate it-DCs using murine and human source materials was investigated in this thesis. In the mouse, the capacity to induce regulatory T cells from naïve CD4 T cell was investigated by examining murine DC subsets harvested from the spleen. In the human, the capacity to induce regulatory T cells from naïve CD4 T cell was investigated by examining circulating DC subsets in human blood as well as human monocyte-derived DCs.

3.1 Dendritic cell distribution in wild type mice

Dendritic cells purified from murine spleens with Flt3 ligand expressing tumors increases the amount of $CD11c^+$ DCs from about 2% in a wild type mouse up to 40% in the spleen within 14 days. We determined the percentages of five different DC subsets among the total $CD11c^+$ DC population in wild type and compared it to Flt3L expressing mice. It was reported that Flt3L expands distinct $CD11c^+$ DCs that show increased immunogenic and tolerogenic capabilities compared to wild type. (Miller *et al.*, 2003) DC subsets are defined as follows: pDC ($CD11c^+$ $PDCA\text{-}1^+$), CD11b ($CD11c^+$ $CD11b^+$ $CD4^-$), CD4 ($CD11c^+$ $CD4^+$ $CD11b^+$), CD8a ($CD11c^+$ $CD8a^+$ $CD4^-$ $CD11b^-$), and triple negative subset ($CD11c^+$ $CD8a^-$ $CD4^-$ $CD11b^-$). Gating strategy for wild type mice is depicted in Fig. 15 and Flt3L mice sorting strategy is shown in methods, whereby the gating strategy for Flt3L mice and wild type mice is very similar.

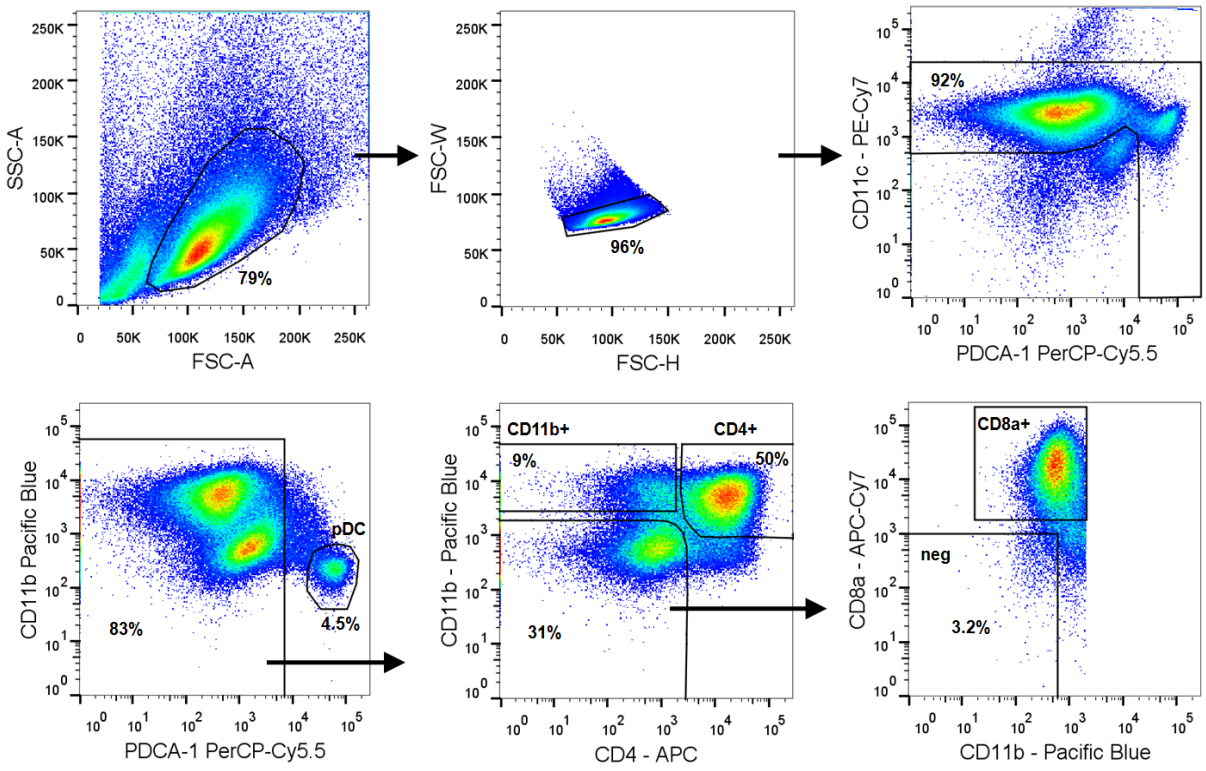

Fig. 15 FACS sorting strategy for murine DC subsets from pre-purified splenic $CD11c^+$ DCs of naïve wild type mice

The amount of extracted splenocytes from Flt3L-treated mice were ~650x10^6, 40% of which were CD11c$^+$ compared to normal spleen which harbor ~80x10^6 splenocytes with 1% CD11c$^+$ (data not shown). During the late stages of the Flt3L-mediated expansion of DCs and their progenitors, the vast majority of DCs are triple negative DCs (in Flt3L expressing mice), which is considered as the most immature subset (Fig. 16A und B). Flt3L shows a major shift towards TN DCs in cell numbers when directly compared to WT. This causes that all other subsets are proportionally higher in WT than Flt3L mice.

In preliminary experiments we determined the precise ratios of DCs:T cells that led to optimal Treg induction. Among the four treatment groups (ctrl, LPS, it-DC, or ctrl-DC+TGFb/IL-2), we noticed that less DCs in coculture with T cells lead to higher Treg induction. This observation can also be confirmed in regard to total Treg cell numbers; 1:5 DC/T cell co-cultures lead to a decreased number of Tregs (23% to 57%) compared to 1:10 (data not shown). Here, we argue that a ratio of 1:10 is sufficient to stimulate the huge majority of T cells in a 96 well round dish by DCs that allows them to expand successfully and a higher DC density could lead to predatory competition between DCs (Fig. 17A). Interestingly, WT and Fltl3l DCs show

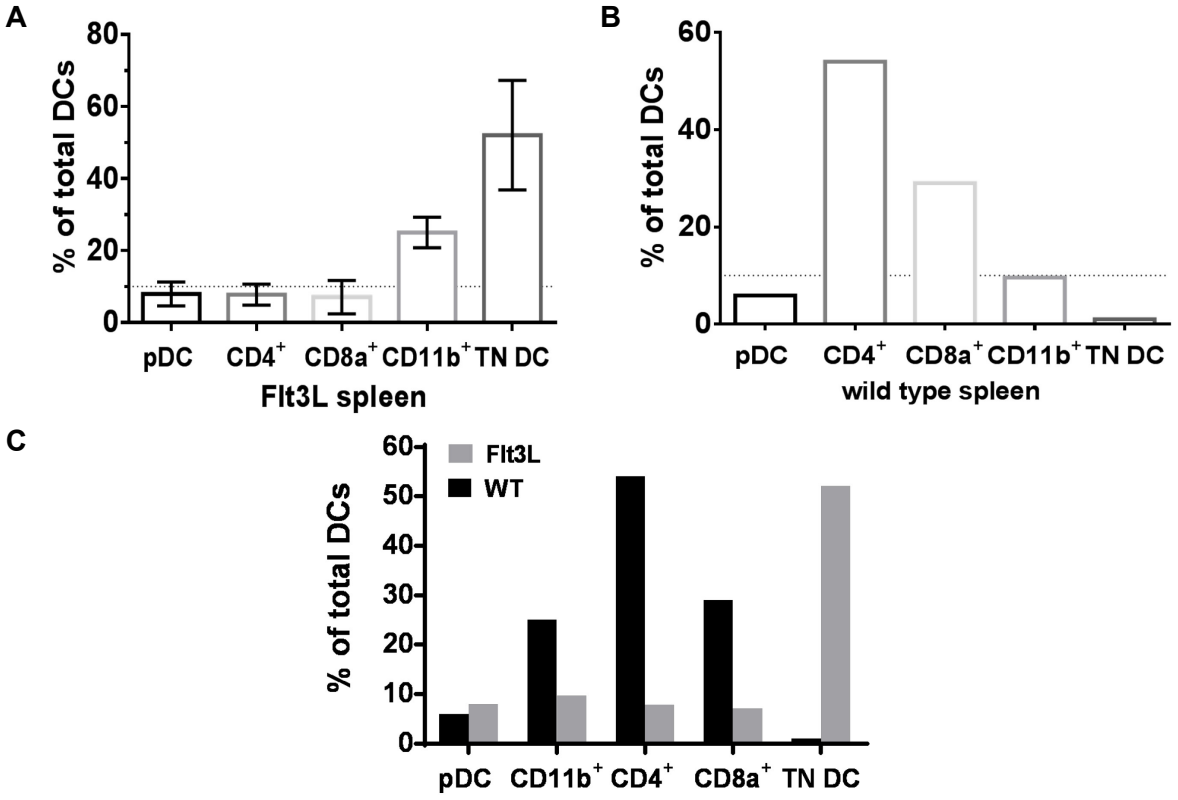

Fig. 16 Splenic DC distribution in wild type **(A)** and Flt3L injected mice after 12 and 14 days of injection, respectively **(B)**. In each experiment spleens were pooled. Data were obtained from two independent experiments for Flt3L mice (n=9). The purity of the populations after sorting was typically >99% as measured by flow cytometry. **(A)** Pools from the majority of DCs in wild type mice are CD4$^+$ (54%), followed by CD8a$^+$ (29%) and CD11b$^+$ (9.6%), whereas pDCs (5.9%) and CD4$^-$ CD8a$^-$ CD11b$^-$ (1%) constitute for less than 10% of the total population. **(B)** Shown is mean±SD of two independent experiments (n=9). pDC (8±3.3), CD11b$^+$ (25±4.2), CD4$^+$ (7.8±2.9), CD8a$^+$ (7±4.6), TN (triple negative; CD4$^-$ CD8a$^-$ CD11b$^-$) (52±15). **(C)** Direct comparison in percentages of splenic DC subsets in wild type (WT) and Flt3L treated mice, independent of total cell numbers.

no significant Treg induction, although WT have a much lower abundance of TN DCs ($CD4^-$ $CD8a^-$ $CD11b^-$; 1% compared to 55% in Flt3L spleens) that was shown by us as one of the two subsets with the highest capacity to induce Tregs in following experiments. However, we assume that other DC subsets such as $CD8a^+$ are able to compensate this. $CD8a^+$ DCs are relatively abundant in WT (29%) and are capable of specifically inducing $Foxp3^+$ $CD25^+$ Tregs to higher extent than other splenic subsets. Still, total Treg cell numbers are decreased in WT (10%) compared to Flt3L (27%) (data not shown). Overall, these results suggest that although $CD11c^+$ WT and Flt3L panDCs differ in fractions of DC subsets, both are capable of inducing similar Treg percentages, but Flt3L panDCs surpass WT panDCs in total Treg cell numbers.

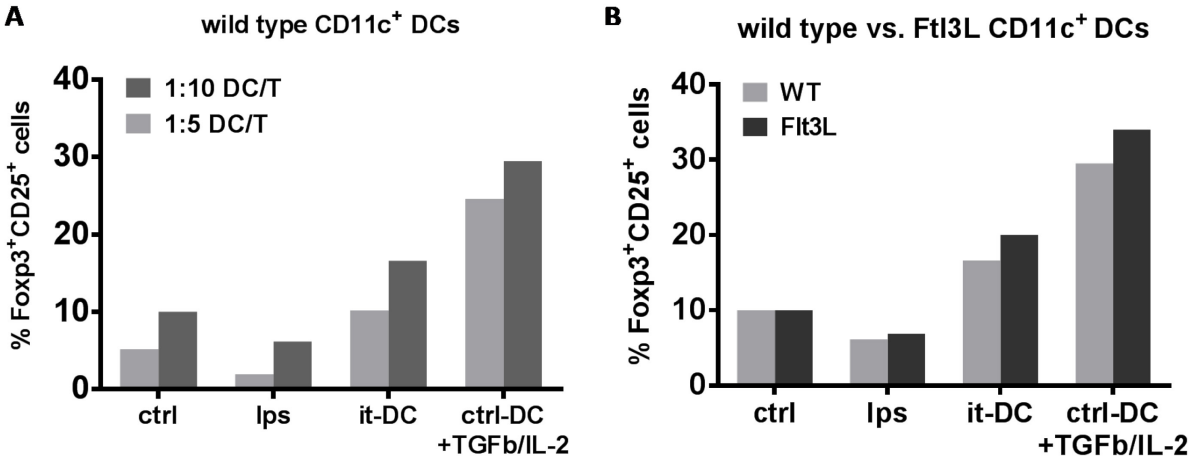

Fig. 17 (A) $Foxp3^+$ $CD25^+$ Treg induction of naïve OT-II cells by $CD11c^+$ splenic DCs from WT in two different DC to naïve T cells ratios (1:5 and 1:10). DCs were pooled from 10 mice. **(B)** $Foxp3^+$ $CD25^+$ Treg induction of naïve OT-II cells by $CD11c^+$ splenic DCs from WT and Flt3L treated mice (ratio 1:10 DCs to naïve T cells). WT and Flt3L (day 9) extracted DCs were treated, cultivated, and cocultured under the same conditions with OT-II cells from the same donor. Cells were gated on single viable cells (viability dye 506).

3.2 Generation of murine induced-tolerogenic DCs

This section describes the *in vitro* experiments performed with $CD11c^+$ DCs (pan DCs) isolated from the mouse spleen in assays examining their capacity to induce $Foxp3^+$ regulatory T cells from naïve CD4 T cells *in vitro* and *in vivo*. These assays were ultimately used to categorize which of the various DC subsets were amenable to "tolerogenic conditioning" and which of the DC subsets were most likely to lead to a therapeutic effect *in vivo*. In this regard, specific it-DC subsets were evaluated *in vivo* in a murine model of allergic asthma, where it-DC subsets were injected to determine their capacity to reduce allergic inflammation.

Firstly, we assessed the Treg induction capacity of rapamycin- and TGFb-treated $CD11c^+$ panit-DCs compared to untreated $CD11c^+$ control DCs. In 14 independent experiments we were able to show a significant difference in the Treg induction capacity of rapamycin- and

TGFb-treated DCs. Since CD11c$^+$ DCs can be divided into phenotypically and functionally distinct subsets, we further investigated if there was a difference in Foxp3$^+$ Treg induction apparent between these subsets.

Secondly, we purified five murine splenic CD11c$^+$ DC subsets in order to assess their potential. These subsets are: CD4$^+$, CD8$^+$, CD11b$^+$, pDC, and CD4$^-$ CD8a$^-$ CD11b$^-$. These experiments uncovered that two subsets, CD8a$^+$ and CD4$^-$ CD8a$^-$ CD11b$^-$, contributed to a greater extent to the Treg expansion observed in previous experiments with CD11c$^+$ panDCs.

Thirdly, we performed *in vivo* experiments with adoptively transferred CD11$^+$ it-DCs and naïve OT-II Foxp3-GFP cells in 2D2 mice, and showed that it-DCs were also able to induce Foxp3$^+$ CD25$^+$ CD4$^+$ expansion *in vivo*.

Finally, the following three groups of it-DCs were tested in a murine model of allergic asthma: pan-DCs, CD8a$^+$, CD4$^-$ CD8a$^-$ CD11b$^-$. Our preliminary results suggest that the CD8a$^+$ DC subset was able to inhibit inflammation compared to untreated mice (no DCs transferred).

Additionally, we performed live cell imaging of DCs and naïve CD4 T cells to visualize the *de novo* induction of Tregs by pan-DCs or sorted DC subsets. To denote Treg induction, we examined the expression of Foxp3$^+$ by visualizing the induction of green fluorescence protein by use of the Foxp3-GFP reporter. Here, we were able to observe morphological differences in Tregs and DCs as well as patterns of interaction between DCs and T cells.

3.2.1 Methodological approaches to study antigen-specific Treg induction

An important methodological aspect of this work was the use of transgenic T cells with a defined antigen specificity to examine antigen-specific regulatory T cell induction. We used OT-II mice, in which all CD4$^+$ T cells bear a genetically defined T cell receptor (TCR) recognizing OVA peptide 323-339. We crossed these mice to Foxp3-GFP reporter mice to allow for ability to directly observe induction of GFP$^+$ Tregs (by flow cytometry or live imaging). Furthermore, we also crossed OT-IIxFoxp3-GFP mice with congenic CD45.1 mice to facilitate the identification and tracking of CD4$^+$ naïve T cells in adoptive transfer experiments, as well as Rag$^{-/-}$ mice to fix the TCR usage (Vβ5.1/5.2 and Vα2). To assure comparability between experiments, the TCR, congenic, Rag, and Foxp3-GFP genotype was determined for all mice in these experiments by flow cytometry of peripheral blood leukocytes (Fig. 18). The mouse colonies maintained for these studies consisted of OT-IIxFoxp3-GFPxCD45.1 Rag^{het}, OT-IIxFoxp3-GFPxCD45.2$^{het/KO}$, and OT-IIxFoxp3-GFPxCD45.1.2 Rag $^{het/KO}$ mice.

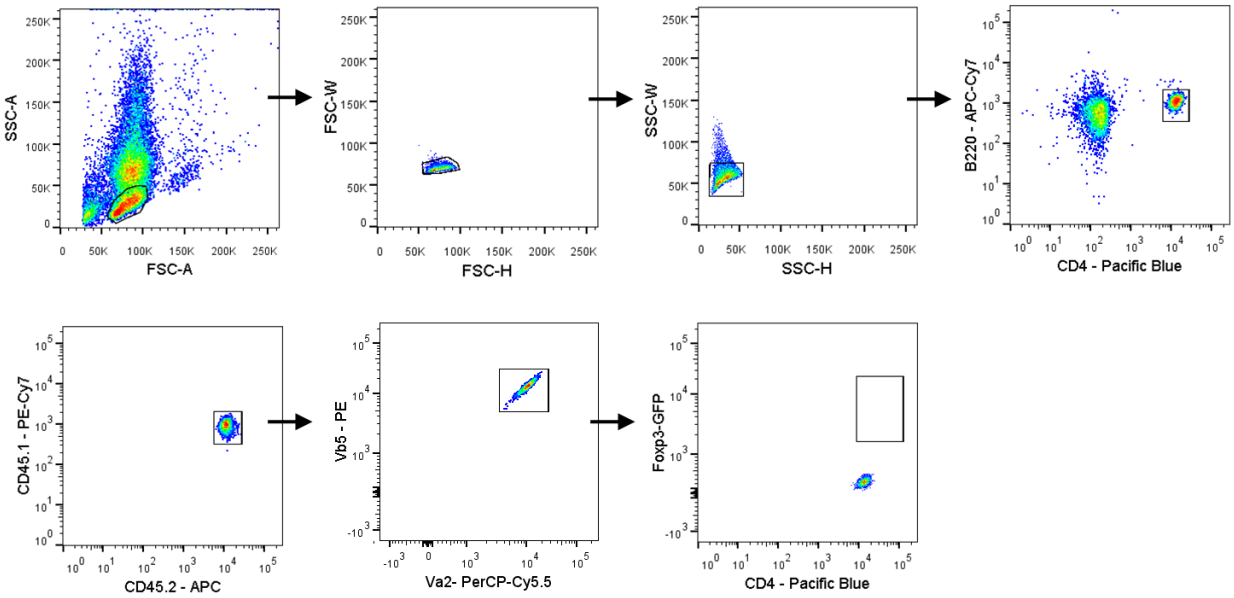

Fig. 18 OT-II Foxp3-GFP colony staining. The first gate encircles lymphocytes, followed by single cell separation and discrimination between B220$^+$ B cells and CD4$^+$ cells T cells. This mouse is CD45.2 positiv and Vb5 and Va2 are expressed on all CD4$^+$ cells. The uniform expression of Vb5 and Va2 on all CD4 T cells, in addition to the lack of Foxp3$^+$ cells confirms that this mouse is a Rag^{KO}.

3.2.2 Murine Foxp3$^+$ CD25$^+$ Treg induction

In our initial experiments, we determined whether rapamycin and TGFb treated CD11c$^+$ DCs (it-DCs) can convert naïve T cells into Tregs, to a greater extent than untreated control DCs or immunogenic DCs. To study this, CD11c$^+$ DCs were extracted from murine FltL3 spleens, pulsed with OVA, and cocultured with naïve CD4$^+$ T cells of OT-II Foxp3-GFP Rag^{het} (or Rag^{KO}) mice. All DCs were pulsed with OVA. Rapamycin and TGFb treated DCs are considered as induced tolerogenic DCs (it-DCs) that are expected to have a superior Treg induction capacity. Rapamycin alters antigen processing and presentation, as well as response to cytokines, growth factors and TLR agonists. (Maldonado and Andrian, 2010) OT-II cells are only capable of recognizing processed OVA with its TCR and interact with peptides bound to MHC on the surface of DCs, but not other antigens. (Robertson *et al.*, 2000) Foxp3-GFP induction is considered as a confirmation of stimulation of naïve T cells by DCs. To exclude dead cells (which can bind non-specifically to antibodies), we stained the cultures with viability dye 506 (Via506) to exclude dead cells (dead cells are Via506$^+$) before gating on single cells and finally on Foxp3$^+$ CD25$^+$ Tregs (Fig. 19). The transgenic Foxp3-GFP is a reliable endogenous reporter to detect Foxp3$^+$ cells with FACS without the need for additional staining of the intranuclear transcription factor Foxp3. However, to verify our findings, cultures were always stained with Foxp3-APC, though no meaningful difference (±5%, data not shown) was detected in total cell numbers or percentages (see FACS gating in Fig. 19). In addition to it-DCs, and control untreated DCs, we also included a group of highly stimulatory or “immunogenic” DCs. In this case, we used LPS stimulation, which

results in DC maturation along with decreased antigen uptake ability, poor migratory function, and high stimulatory activity of DCs. Therefore, LPS stimulated DCs are considered "immunogenic" DCs with the lowest Treg induction capacity in our experimental DC groups. (Granucci *et al.*, 1999) Furthermore, as a positive control, we used a combination of IL-2 and TGFb that was added to untreated control DCs to potentiate the capacity of Treg induction through the direct action of IL-2 and TGFb. The positive control is expected to have maximum Treg induction capacity due to an unphysiological excess of IL-2 and TGFb; cytokines that drive Treg expansion.

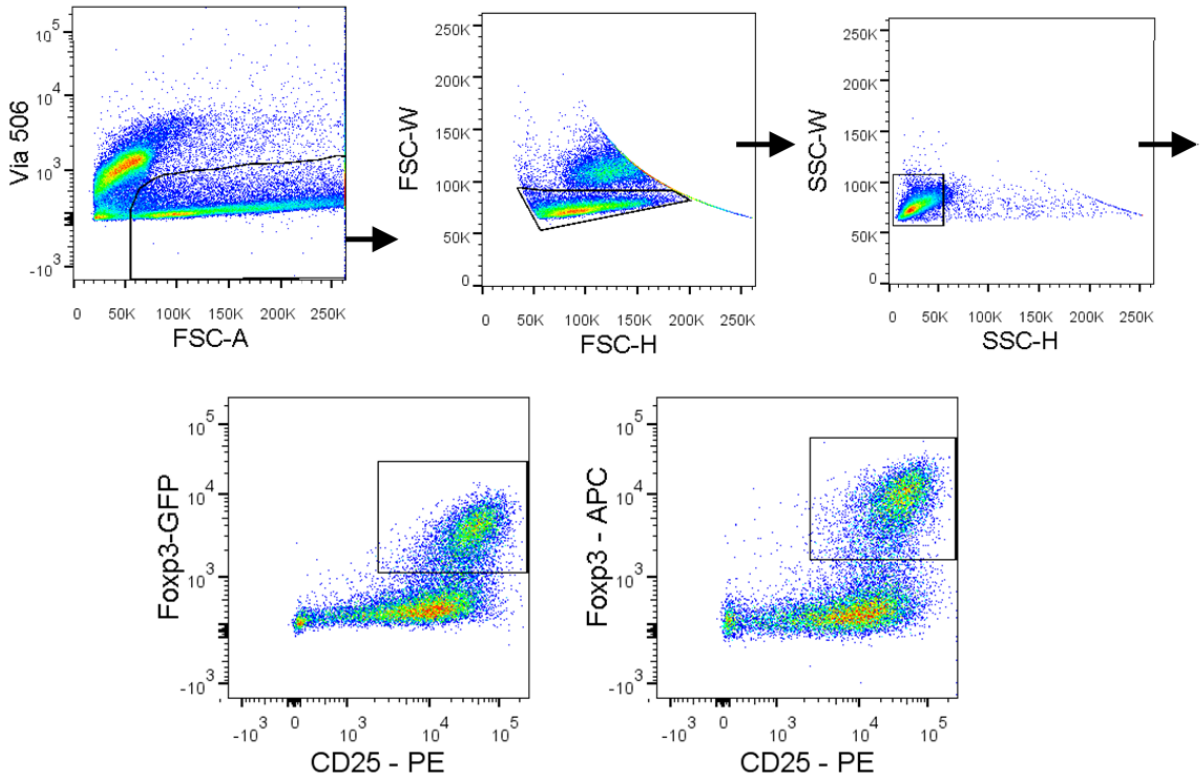

Fig. 19 FACS gating strategy for Foxp3$^+$ CD25$^+$ Tregs. Cultures were harvested after 5.5 days and stained with viability dye (Via 506) in order to discriminate between viable and dead cells. It was shown that the endogenous Foxp3-GFP reporter was not inferior to intranuclear staining with Foxp3-APC, though was used as a control in all experiments. This FACS plot is representative for gating on Foxp3$^+$ CD25$^+$ CD4$^+$ Tregs.

3.2.2.1 Treg induction by splenic panDCs

Our experiments revealed a highly significant increase in Treg induction capacity of CD11c$^+$ it-DCs over control DCs and LPS treated DCs in percentages of activated Foxp3$^+$ CD25$^+$ CD4$^+$ T cells. it-DC Treg induction (24.5%) was similar to positive control (28%). Likewise, LPS DC induction (4%) was comparable in induction efficiency to untreated control DCs (4%). (Fig. 20A). Total Foxp3$^+$ Treg cell numbers were also increased in it-DCs (2.4-fold) and positive control (7.9-fold) compared to control, where the LPS-DC group (0.9-fold) showed a minor reduction what was expected (Fig. 20B). The difference in total cell numbers is also a result of more unspecific activation and proliferation in the positive control due to the addition

of IL-2 and TGFb. Contrary, it-DCs which are suggested to suppress unspecific activation of T cells and $CD8^+$ T cells lead to lower cell numbers. In positive control, the unrestricted proliferation of other T cells may lead to even higher cytokine levels that cause additional expansion of Tregs, though this may not be considered a physiological response. Tregs induced by it-DCs were increased in every single experiment over ctrl DCs. Fig. 20D reveals that individual experiments that resulted in lower Treg percentages; it-DCs still surpass control DC Treg induction.

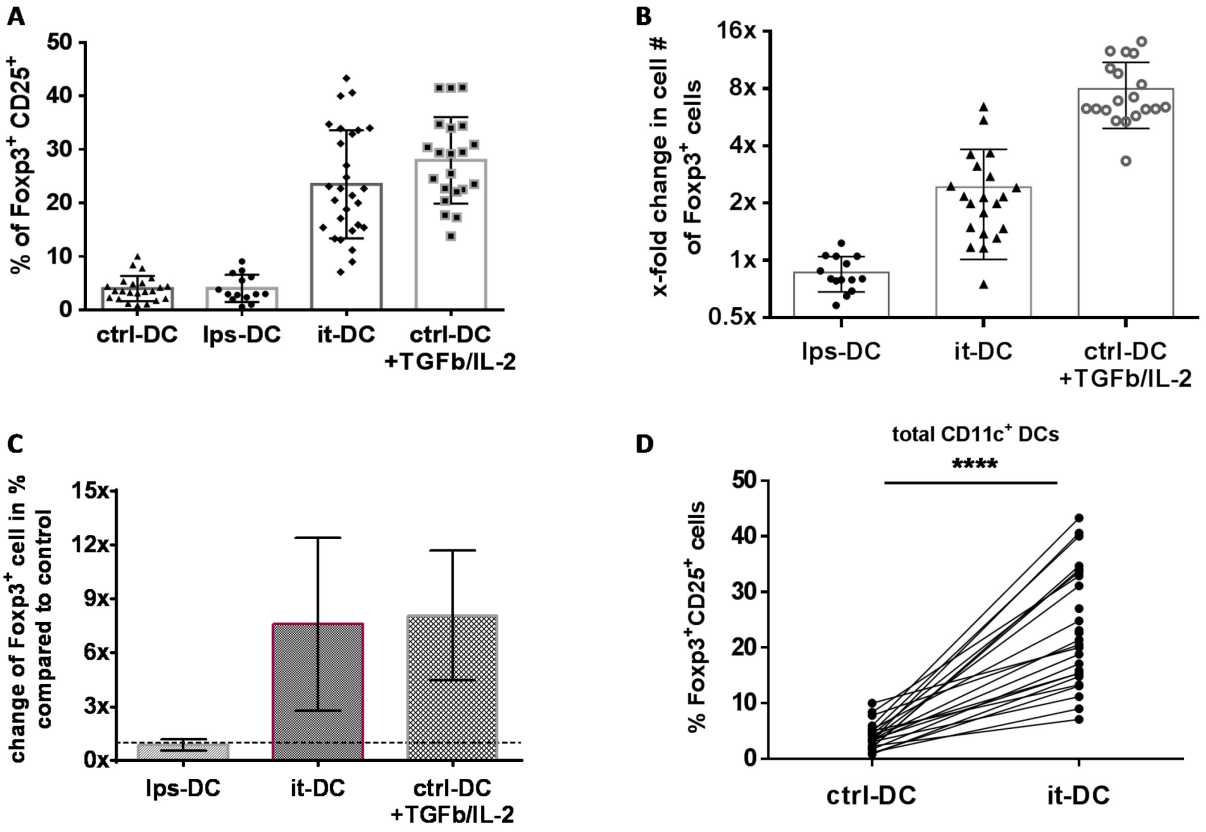

Fig. 20 $Foxp3^+$ $CD25^+$ Treg induction by total $CD11c^+$ splenic DCs (panDCs). Data obtained from 14 independent experiments. Gated on single viable cells (viability dye Via 506). control-DC + TGFb/IL-2 serves as positive control and indicates the maximum Treg induction due to the addition of TGFb and IL-2. **(A)** % of $Foxp3^+$ Tregs of total viable cells. ctrl-DC (4% ± 2.4), LPS-DC (4% ± 2.5), it-DC (24.5% ± 10.1), control-DC + TGFb/IL-2 (28% ± 8). There is also no significant difference between it-DCs and ctrl-DC+TGFb/IL-2 (paired t-test; $p \geq 0.147$, n=21, 95% CI -1.38 to 8.56). This means that it-DCs could induce Tregs as well as the positive control in percentages of the total culture. **(B)** x-fold change of $Foxp3^+$ cell numbers compared to untreated control DCs (1x). LPS-DCs (0.9-fold) showed on average an inferior induction of $Foxp3^+$ Tregs than control DCs. However, it-DCs and positive control showed on average a 2.4-fold and 7.9-fold increase in Treg cell numbers compared to control, respectively**. (C)** Depicted is the x-fold change in $Foxp3^+$ $CD25^+$ Tregs compared to control in each experiment. The dotted line indicates the $Foxp3^+$ $CD25^+$ Tregs in control (1x) (mean ± SD; median). LPS-DCs (0.9 ± 0.3; 0.8) induces less Tregs compared to control, whereas it-DCs (7.6 ± 4.8; 6.7) revealed a significant capacity that was comparable with ctrl-DC + TGFb/IL-2 (8.1 ± 3.6; 6.9). **(D)** There is a highly significant difference between ctrl-DCs and it-DCs, whereby it is proven that $CD11c^+$ it-DC cultures show an increase in percentages of $Foxp3^+$ $C25^+$ $CD4^+$ T cells. Statistical signficance derived from a paired t-test **** $p < 0.0001$, n=22, 95% CI 14.6 to 24.

3.2.2.2 Treg induction by DC subsets

In order to assess the potential of different DC subsets, we isolated CD11c$^+$ pan DCs from spleen with magnetic labeling and further sorted the subsets into CD4$^+$, CD8$^+$, CD11b$^+$, pDC, and CD4$^-$ CD8a$^-$ CD11b$^-$ by flow cytometry. We analyzed Treg induction of each of these subsets in up to four independent experiments. In all cases, each subset was untreated (i.e. ctrl DC), LPS-treated DCs, rapamycin plus TGFb treated DCs (it-DCs), and positive control (ctrl-DC + TGFb/IL-2). All cultures, except pDCs, showed good growth and viablity in all cultures. Foxp3$^+$ CD25^{hi} Tregs are a clearly defined population (Fig. 21). Significant differences in Treg activation have been identified between it-DC subsets. Triple negative DCs and CD8a$^+$ DCs were identified as the most potent it-DCs that give rise to high percentages of Tregs.

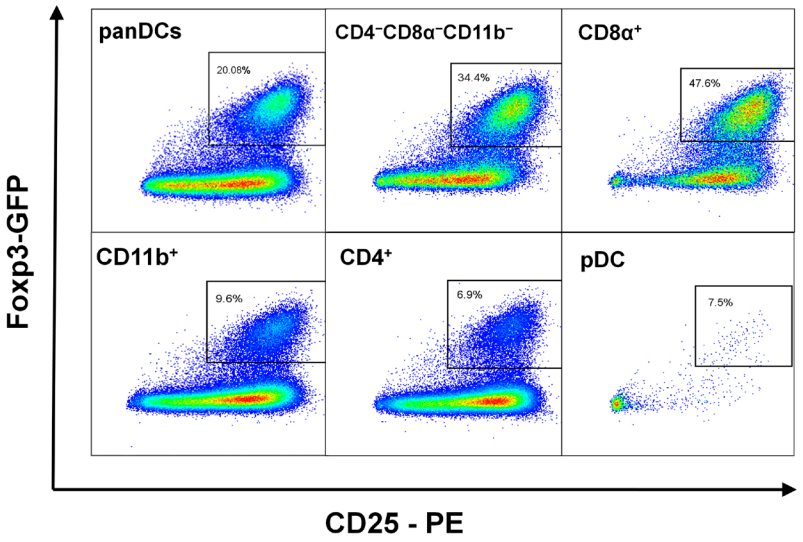

Fig. 21 Gated cells are Foxp3$^+$ CD25$^+$ Tregs. The CD4$^-$ CD8a$^-$ CD11b$^-$ and CD8a$^+$ subsets show a homogenous population of Tregs with a high percentage of Tregs and high total Treg cell numbers. Very low total viable cell number in pDC cultures are indicative of that naïve T cells did not develop properly. Therefore, it-pDCs show also an inferior effect of it-DC reprogramming.

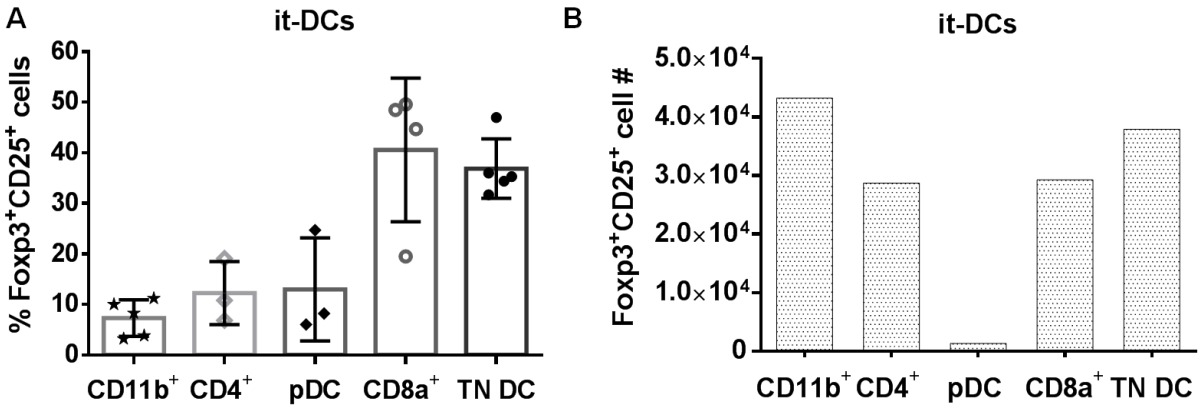

Fig. 22 Foxp3$^+$ CD25$^+$ Treg induction by murine spleen it-DC subsets. Naïve Foxp3-GFPxOT-II CD4$^+$ cells were stimulated in a coculture with ovalbumin (100ng/ml) pulsed DCs at a 1:10 DC/T cell ratio. DCs were purified from groups of 2 to 5 mice. All subsets (CD4$^+$, CD8$^+$, CD11b$^+$, pDC, CD4$^-$ CD8a$^-$ CD11b$^-$) are it-DCs. Cells were stained and induction of Foxp3$^+$ CD25$^+$ CD4$^+$ T cells was analyzed at day 5.5. **(A)** Results are depicted as percentages of murine Foxp3$^+$ CD25$^+$ Tregs from the total live cell population from three to four independent experiments, because not all subsets were analyzed in all experiments. Treg induction by DC subset (mean ± 1 SD): CD11b$^+$ (7.3 ± 3.6%), CD4$^+$ (12.3 ± 6.2%), pDC (13 ± 10.2%), CD8a$^+$ (40.6 ± 14.2%), TN (CD4$^-$ CD8a$^-$ CD11b$^-$) (36.9 ± 5.9%). **(B)** Foxp3$^+$ CD25$^+$ Treg cell numbers are derived from a single experiments, but are representative in ratios in all experiments. It can be clearly seen that pDCs have very low numbers of Tregs, but also very low total viable cell numbers (data not shown). CD11b$^+$ show in all experiments the highest number of Tregs, followed by TN, CD8a$^+$ and CD4$^+$ it-DCs.

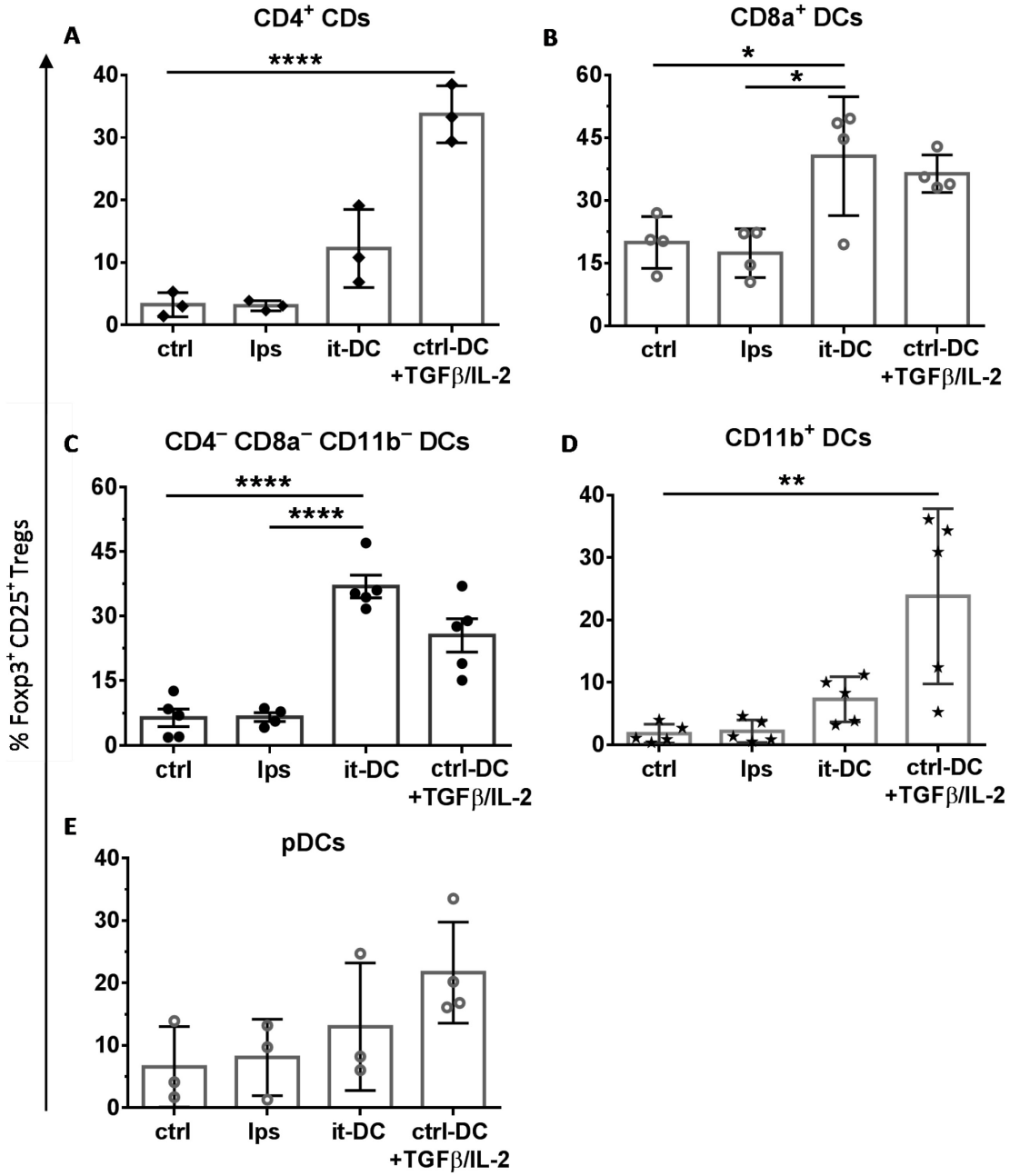

Fig. 23 Foxp3$^+$ CD25$^+$ CD4$^+$ Treg induction by murine spleen DC subsets. Naïve Foxp3-GFPxOT-II CD4$^+$ cells were cocultured with ovalbumin (100ng/ml) pulsed DCs at a 1:10 DC/T cell ratio. Four conditions were used for each DC subset: untreated control DCs (ctrl-DC), DCs treated with lipopolysaccharide (1 µg/ml) (LPS-DC) as negative control, rapamycin (10 ng/ml) + TGFb (2 ng/ml) treated DCs (it-DCs), and ctrl-DCs with TGFb (20ng/ml) + IL-2 (20 U/ml) as positive control. **(A)** CD4$^+$ it-DCs (12.25±6.25) are inducing more Tregs in cultures in percentages compared to ctrl (3.2±1.9) and LPS (3±0.8%). However, the difference between the positive control group and it-DCs was substantial and CD4$^+$ it-DCs show much lower Treg induction compared to CD8a and TN. **(B)** CD8a$^+$ DCs show a high induction in the it-DC group (40.6±14.2) and even outnumber the positive control (36.4±4.5), though also ctrl and LPS show the highest induction measured among all subsets. **(C)** CD4$^-$ CD8$^-$ CD11b$^-$ it-DCs showed a significantly increase of Foxp3$^+$ CD25$^+$ cells in culture and also outnumbered the positive control in all experiments, but not in total Treg cell numbers (data not shown). **(D)** CD11b$^+$ ctrl and LPS DCs show very low intrinsic activity to induce Tregs, though the it-DC subset shows an equal number of Tregs in cell numbers compared to CD8a and the triple negative subset, but the graph depicts that Treg are not specifically induced by CD11b$^+$ it-DCs (7.3±3.6% Tregs of all viable cells in culture). **(E)** Though it seems that pDCs induce a small percentage of Foxp3$^+$ Tregs, it cannot be concluded that pDCs are able to induce Tregs, because pDCs show a very low percentage of viable cells in culture (see Fig. 21). We cannot conclude that pDCs are unable to induce Tregs, but our culturing conditions lead to inferior induction of Tregs and viability of all cells in pDC cultures. No statistical significant difference between ctrl-DCs and it-DCs or positive control (paired t-test, 95% CI) was detected in pDCs. All figures: significance levels between ctrl, LPS or it-DC with the positive control is only indicated if no other significance was detected and not all significance levels are necessarily noted for ctrl-DC + TGFb/IL-2.

However, in total cell numbers CD11b$^+$ it-DCs generate the highest numbers of Tregs, followed by TN and CD8a it-DCs (Fig. 22B). A more detailed analysis of all experimental groups of the different it-DC subsets revealed that CD8a$^+$ and TN it-DCs show a significantly increased capacity to selectively induce Tregs. Due to the stimulating culture conditions through the addition of TGFb and IL-2, the positive control DCs excelled in total Treg cell numbers (data not shown), but CD8a$^+$ and TN DCs surpassed the positive control in percentages of Tregs on average. This is suggestive of a selective induction mechanism together with a suppression of other CD4$^+$ T cells. This capability is not shared by all DC subsets, but rather restricted to CD8a and triple negative DCs that is unveiled by a short-time treatment with rapamycin. Though CD11b$^+$ it-DCs were able to induce high cell numbers of Tregs that even outnumbered all other subsets (Fig. 22B), they failed in showing a specific

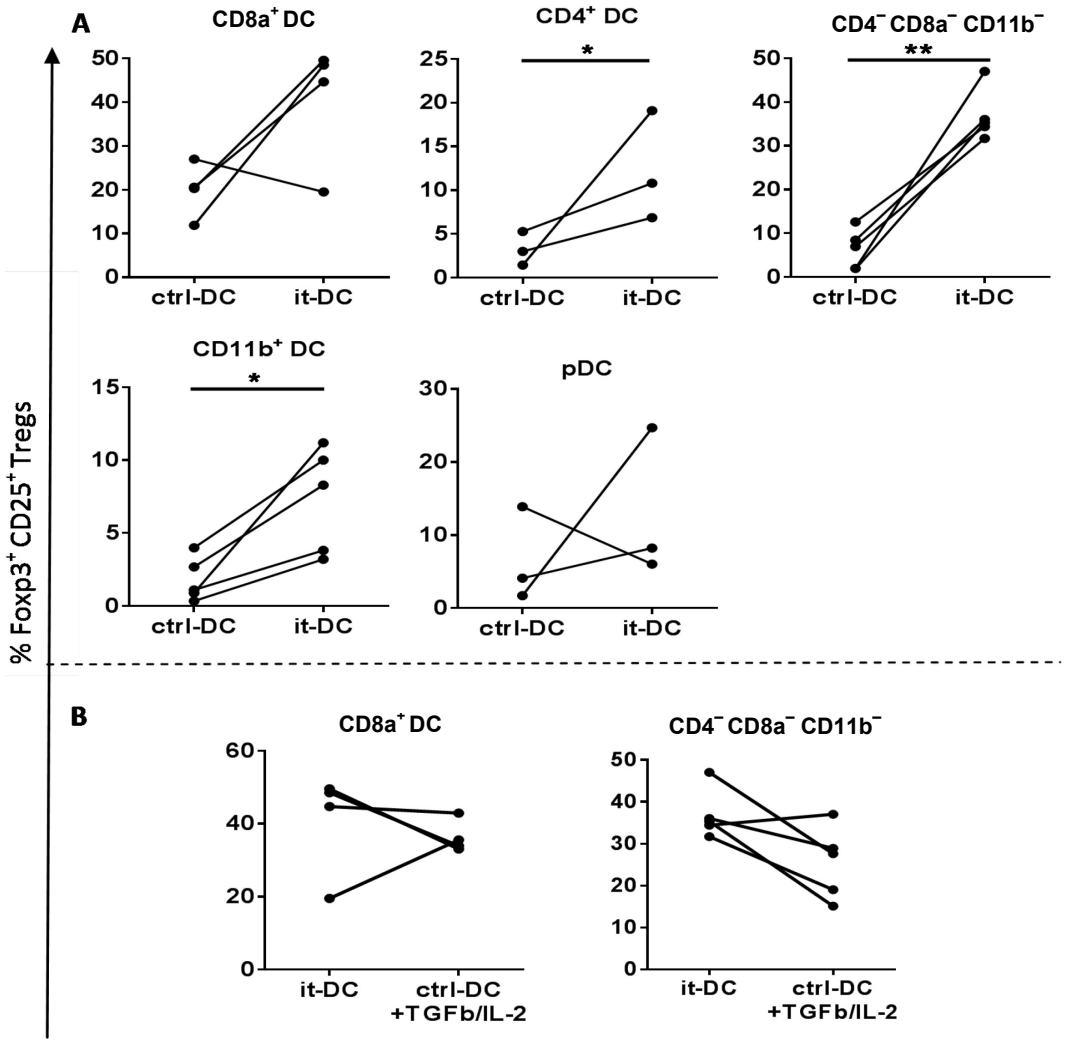

Fig. 24 (A) Direct comparison between control and rapamycin treated DC subsets (paired t-test): CD4$^-$ CD8a$^-$ CD11b$^-$ ($p \leq 0.0017$, n=5, 95% CI 19.12 to 41.91). CD4$^+$ DCs ($p \leq 0.032$, n=3, 95% CI 4.56 to 38.4). CD11b$^+$ DCs ($p \leq 0.0164$, n=5, 95% CI 1.67 to 9.34). pDC and CD8a$^+$ ($p < 0.05$). **(B)** Differences between it-DC subsets and positive control. There is no significant difference that CD4$^-$ CD8a$^-$ CD11b$^-$ it-DC cultures and CD8a$^+$ it-DCs induce more Foxp3$^+$ CD25$^+$ Tregs compared to ctrl-DC + TGFb/IL-2 (paired t-test with n=5, $p \geq 0.0549$, 95% CI (-23.10 to 0.3804) and ($p \geq 0.6151$, n=4, 95% CI 28.11 to 19.71), respectively.

induction of Foxp3^{+} CD25^{hi} cells in terms of Treg percentages of the total population.

Additionally, we analyzed all subsets for each experiment independently between control and rapamycin + TGFb treatment to evaluate the difference of these groups better. In the majority of all individual experiments over all subsets, it can be assumed that the higher ctrl-DC Treg % are, the higher it-DCs % are as well. Moreover, it helped us to observe how outliers distort significance due to comparatively low number of experiments. Thereby, significance levels were assessed with a paired t-test (Fig. 24). Interestingly, CD8a and TN it-DCs exceeded positive controls on average, though the added cytokine cocktail (TGFb and IL-2) should induce maximal Treg induction in cultures. However, a direct comparison of it-DCs and positive control with a paired t-test revealed no statistical difference (Fig. 24B).

3.2.2.3 Macrophages and lung-DCs

In addition to examining DC-mediated induction of Tregs, we also considered whether macrophages could induce Tregs. To do so, we compared the *in vitro* induction of Foxp3^{+} CD25^{+} CD4^{+} Tregs from naïve CD4 T cells, by either splenic DCs or lung-resident macrophages and lung-resident DCs. This experimental design compared the potency of splenic DCs versus unstimulated macrophages based on a recent publication that suggested lung macrophages have intrinsic tolerogenic activity and are capable of inducing Tregs (Soroosh *et al.*, 2013). Lungs from 10 naïve murine lungs were perfused and lavaged (BAL) to exclude circulating cells and bronchoalveolar cells to specifically determine lung tissue resident macrophages and DCs. All macrophages and DCs were treated under the same conditions and cocultured with OT-II cells from the same donor. No IL-2 was added to the cultures to sustain the highest degree of intrinsic Treg induction capacity (see methods).

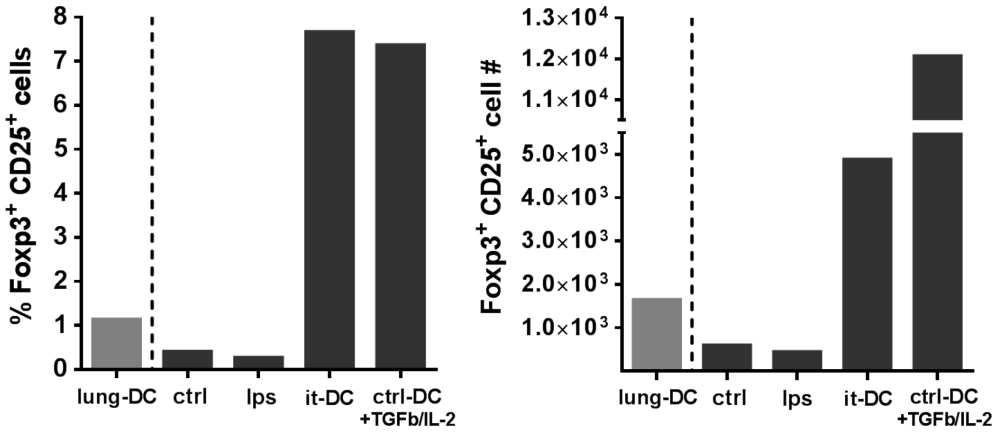

Fig. 25 The difference of Foxp3^{+} Tregs in % of all viable cells and in total cell numbers between lung-DCs and our "standard" cultures (ctrl, LPS, it-DC, ctrl-DC + TGFb/IL-2) are depicted. CD11c^{+} lung-DCs may have a higher intrinsic activity to drive naïve T cells into the Treg pathway compared to total CD11c^{+} DCs. A direct comparison between the different splenic DC subsets and the two main lung-DCs subsets are a necessary measure to determine a significant difference.

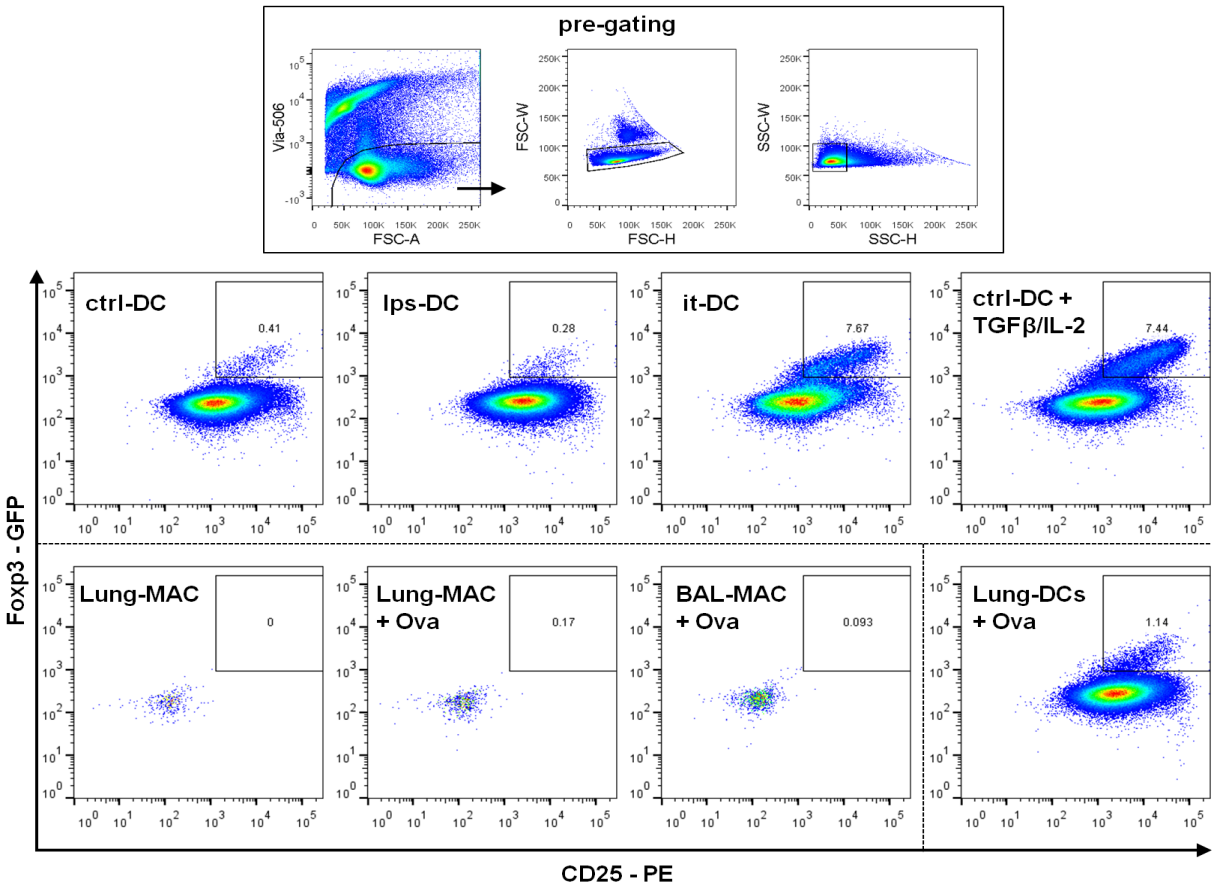

Fig. 26 Gating (example for pre-gating from lung-DCs) of Foxp3$^+$ CD25$^+$ Tregs that were induced by murine CD11c$^+$ spleen DCs (ctrl-DCs, LPS-DC, it-DC, ctrl-DC+TGFb/IL-2 as in previous experiments, see methods), non ovalbumin and ovalbumin pulsed CD11c$^+$ Siglec-F$^+$ MHC-II$^+$ CD45$^+$ lung resident tissue macrophages (Lung-MAC and Lung-MAC + Ova, respectively), bronchoalveolar lavage (BAL) collected macrophages pulsed with Ovalbumin (BAL-MAC + Ova), and CD11c$^+$ CD45$^+$ Siglec-Flo MHC-II^{lo} lung resident tissue DCs (Lung-DCs + Ova). We gated on CD11c$^+$ CD45$^+$ cells to encompass all DC and macrophage populations. Gated cells are Foxp3$^+$ CD25$^+$ Tregs. Splenic CD11c$^+$ DCs and lung-DCs showed good growth in culture (52% to 66% in splenic DCs and 63% in lung-DCs of viable cells of total cell count). However, only 3.3% (Lung-MAC + Ova), 3.5% (Lung-MAC), and 6% of the BAL-MAC cells in culture were considered as viable cells. Under these conditions, macrophages were not able to induce Tregs to a meaningful quantity. However, lung-DCs (1.14%) showed a increased capacity compared to ctrl-DCs (0.41%) in the induction of Foxp3$^+$Tregs, though if this difference is statistically significant would need to be determined in further experiments.

In this experiment, we were not able to detect an increased Foxp3$^+$ Treg induction by lung macrophages from BAL or lung tissue resident macrophages compared to DCs (Fig. 26). However, lung tissue resident DCs showed cell expansion and Treg induction comparable to splenic DCs (1.14%) with minor rise in Tregs induction compared to control DCs (0.41%). Due to the low abundance of lung tissue resident cells and the experimental effort to isolate those, it was not practicable with current protocols to investigate this subset for it-DC reprogramming. Here, we were only able to cultivate one culture out of lung tissue from 10 mice with a reasonable amount of lung-DCs. Moreover, we did not distinguish between the two major lung cDC populations CD103$^-$ CD11b^{hi} and CD103$^+$ CD11b^{lo}. (Kopf *et al.*, 2014)

No further experiments were conducted with macrophages due to the low Treg stimulation. Though, lung DCs, contrary to macrophages, showed promising Treg induction, we did not further investigated the different lung cDC populations due to methodical restrictions.

3.2.3 *in vivo* induction of regulatory T cells

Our *in vitro* assays clearly demonstrated that it-DCs were able to selectively induce Tregs from naïve CD4 T cells in terms of both percentage and absolute number. However, the induction of bona fide Tregs *in vivo* is far more challenging. Here, we examined the *in vivo* induction of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells by adoptively transferred OVA-pulsed it-DCs in transgenic 2D2 mice. The TCR of 2D2 mice is specific and limited to recognize the myelin oligodendrocyte glycoprotein (MOG). We chose this model because T cells from these mice are not capable of recognizing ovalbumin presented by OVA-pulsed DCs, and thus will not be capable of responding to the DCs. (Bettelli *et al.*, 2003). Furthermore, in this model, only the adoptively transferred FACS purified naïve CD45.1.2 OT-II Foxp3-GFP T cells would recognize the OVA-pulsed DCs. This gives us the added certainty that our transferred naïve CD45.1.2 $CD4^+$ T cells are the only T cells in the host 2D2 mice that are capable of recognizing ovalbumin antigens from our ovalbumin pulsed DC groups (control,- LPS treated,- and it-DCs).

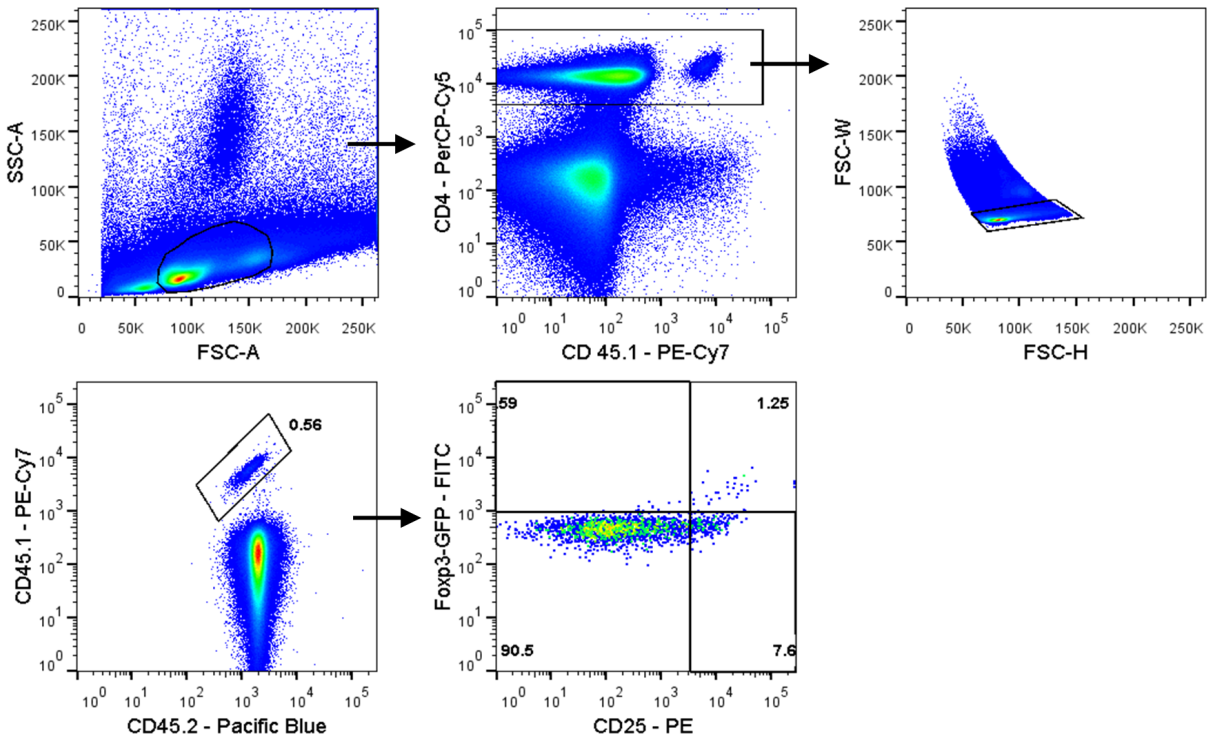

Fig. 27 Gating of $Foxp3^+$ $CD25^+$ $CD4^+$ $CD451.2^+$ cells from processed popliteal,- cervical,- draining popliteal lymphnodes and spleen from 2D2 mice (see methods). Adoptively transfered naïve T cells were isolated from a male CD45.1.2 OT-II Rag^{KO} Foxp3-GFP mouse. 2D2 recipients were male CD45.2 transgenic mice. 200x10^3 naïve OT-II cells were injected i.v.in each mouse. The CD45.1.2 cells are from the transfered OT-II cells and show good proliferation in the course of 7 days. Therefore, the transfered DCs (foot pad (left), intra nasal, and i.v.) were not only able to stimulate naïve T cells *in vivo*, but to induce $Foxp3^+$ Tregs.

2D2 mice cells are CD45.2., whereas adaptively transferred T cells are CD45.1.2, allowing us to easily identify the transferred OT-II cells in FACS (Fig. 27). First, *in vivo* activation and expansion of OT-II cells was detected in all investigated tissues (popliteal LNs, cervical LNs spleen, and blood) (Fig. 28C). Second, $CD11c^+$ it-DCs (pan-DCs) produced the highest cell numbers of $Foxp3^+$ $CD25^+$ Tregs in all examined tissues compared to control and LPS treated DCs. No Tregs were detected in blood (Fig. 28A). Third, specific induction of $Foxp3^+CD25^+$ cells in regard to $Foxp3^-$ $CD25^+$ T cells (ratio) compared to ctrl DCs was observed in cervical LNs and to a lesser extent in the right popliteal LN (non-draining LN).

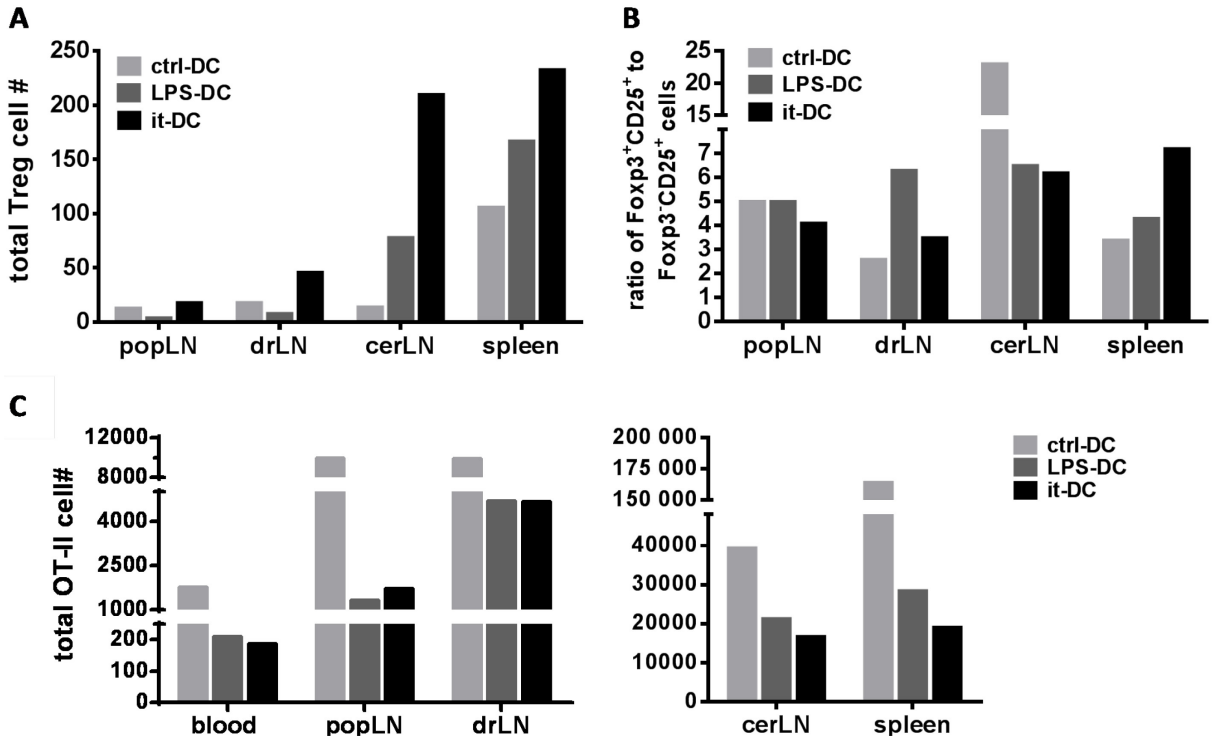

Fig. 28 $Foxp3^+$ $CD25^+$ $CD4^+$ CD45.1.2 induction *in vivo* by control-DCs (ctrl-DC), LPS treated DCs (LPS-DC), and rapamycin + TGFb treated DCs (it-DCs). Cells were FACS analyzed from popliteal lymph nodes (LN), cervical LNs, spleen, and blood. DCs were injected in the left foot pad and i.v. (retro-orbital). Therefore, the left popliteal lymph node is here referred to as the draining lymph node (drLN). The right popliteal lymph node is here referred to as popLN and the cervical lymh node as cerLN. 2.5x10^6 cells were analyzed by FACS of each sample (less cells were analyzed from blood). No Tregs were detected in blood. **(A)** Total cell numbers are extrapolated from total cell counts of collected tissues such as spleen with total cell numbers between 67 to 83 mio cells. it-DCs showed a superior Treg induction capacity. Most Tregs resided in the spleen, but high numbers of Tregs have been detected in cerLN; also taken into consideration that cervical LNs had up to 5.6-fold less total cells compared to spleen. DCs and naïve OT-II cells were injected i.v. retro-orbital. Therefore, it is not surprising that a high number of $Foxp3^+$ cells could be detected in proximity (note: cerLN) to the site of injection. Additionally, a higher number of Tregs have been detected at the side of DC injection for it-DCs at the left draining popliteal lymph node (drLN) compared to the right popLN where no DCs have been injected in the foot pad. **(B)** We compared cells numbers for $Foxp3^+$ $CD25^+$ and $Foxp3^-$ $CD25^+$ T cells. The higher the ratio, the more unspecific was the induced T cell activation. This means that a higher ratio reveals a non specific T cell activation, whereas a lower ratio shows that the induction is specific for $Foxp3^+$ Tregs. The cerLN group shows a major difference between the control group and it-DCs, but also LPS-DCs. This tell us that ctrl-DCs induced an effective T cell response (see also total cell numbers of OT-II cells) and were able to stimulate the adaptively transferred naïve CD45.1.2 T cells, but were not able to induce specifically $Foxp3^+$ Tregs. However, LPS-DC also have a similar ratio such as it-DCs in the cerLN, but show inferior numbers of total $Foxp3^+$ Tregs. Contrary, in spleen the response was less Treg specific compared to control DCs. **(C)** Though the control group outnumbered the other groups in regard to OT-II cell proliferation in all tissues including blood. However, Treg cell numbers were higher in the it-DC group (see (A)).

Contrary, activation of T cells was more unspecific in spleen where a higher percentage of $CD25^+$ activated OT-II cells were not $Foxp3^+$ and thus are not considered as Tregs (Fig. 28B).

Here, we were able to demonstrate that it-DCs are stimulating Treg differentiation *in vivo*. Therefore, the next step was to determine if one DC subsets is capable of inhibiting an allergic inflammatory response in mouse models of asthma

3.2.4 it-DC therapy in OVA-based asthma models

Asthma is a chronic inflammatory disease of the airways as a result of continued or intermittent allergen exposure that usually occurs via inhalation. Mouse models of acute allergic responses to inhaled allergens, such as artificial asthma-like reactions with ovalbumin (OVA), are used to understand and investigate the underlying mechanisms to control the allergic inflammation. (Nials and Uddin, 2008)

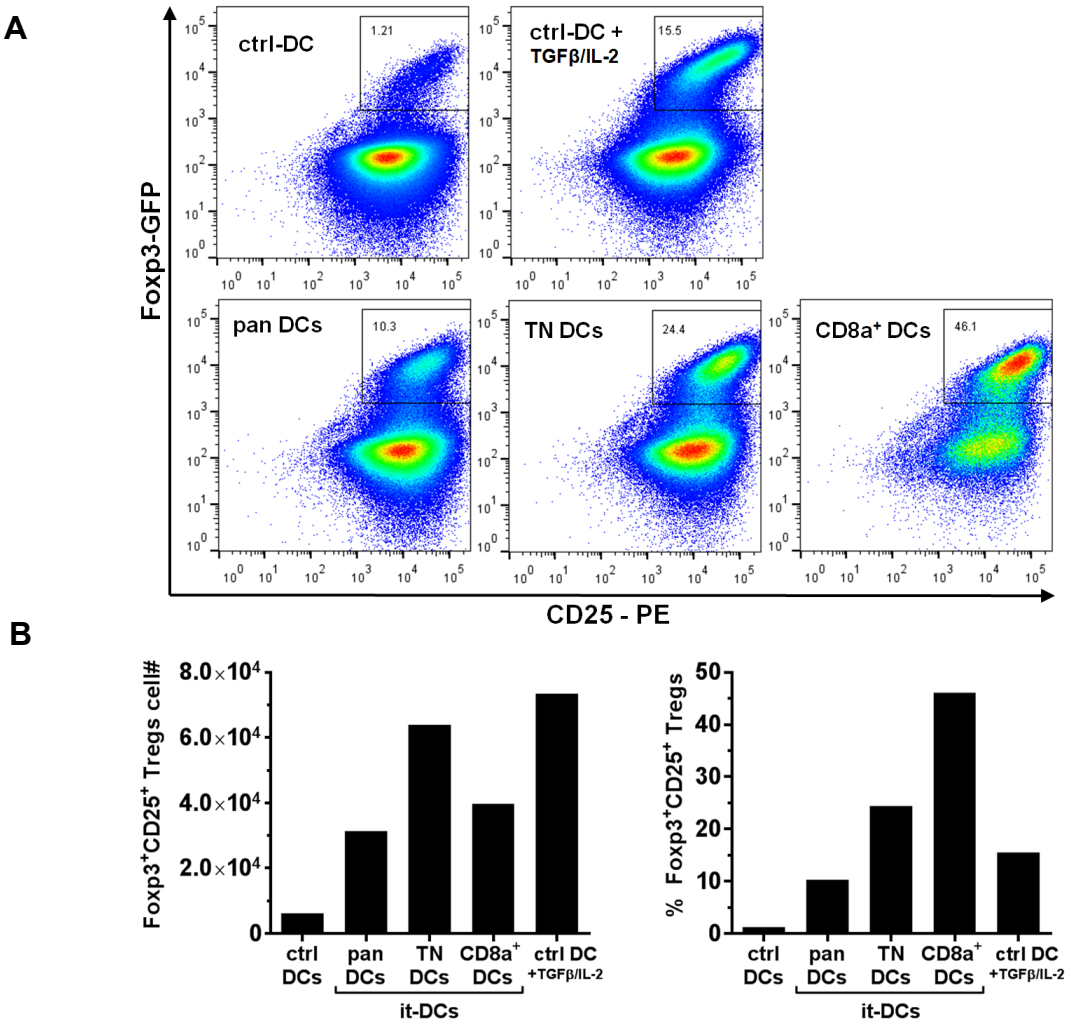

Fig. 29 *in vitro* it-DC quality control. **(A)** Depicted are $Foxp3^+$ $CD25^+$ $CD4^+$ Tregs gated in FACS of three rapamycin + TGFb treated and ovalbumin pulsed DC subsets; $CD11^+$ DCs (pan DCs), triple negative DCs (TN DCs; $CD11c^+$ $CD8a^-$ $CD4^-$ $CD11b^-$), and $CD8a^+$ DCs ($CD8a^+$ $CD11c^+$ $CD4^-$ $CD11b^-$). The quality control also includes both non-rapamycin treated control DCs (ctrl-DCs) and the positive control (ctrl-DC+TGFb/IL-2). **(B)** The $CD8a^+$ subset performed best of all three subsets in terms of $Foxp3^+$ $CD25^+$ Treg induction (46.1% of viable $CD4^+$ T cells), followed by TN DCs (24.4%), pan DCs (10.3%), positive control (15%) and ctrl-DCs (1.21%). However, TN DCs (63,950) surpassed $CD8a^+$ (39,700), pan DCs (31,350), and ctrl-DCs (6,200) in cell numbers.

Foxp3$^+$ CD4$^+$ cells have been previously shown to mediate the inhibition of allergic airway inflammation, suggesting a role for these cells in controlling allergic asthma. (Ostroukhova et al. 2004) The aim of this experiment was to determine if the administration of it-DCs inhibits allergic airway inflammation by creating immunological tolerance through the induction of Foxp3$^+$ Tregs by it-DCs.

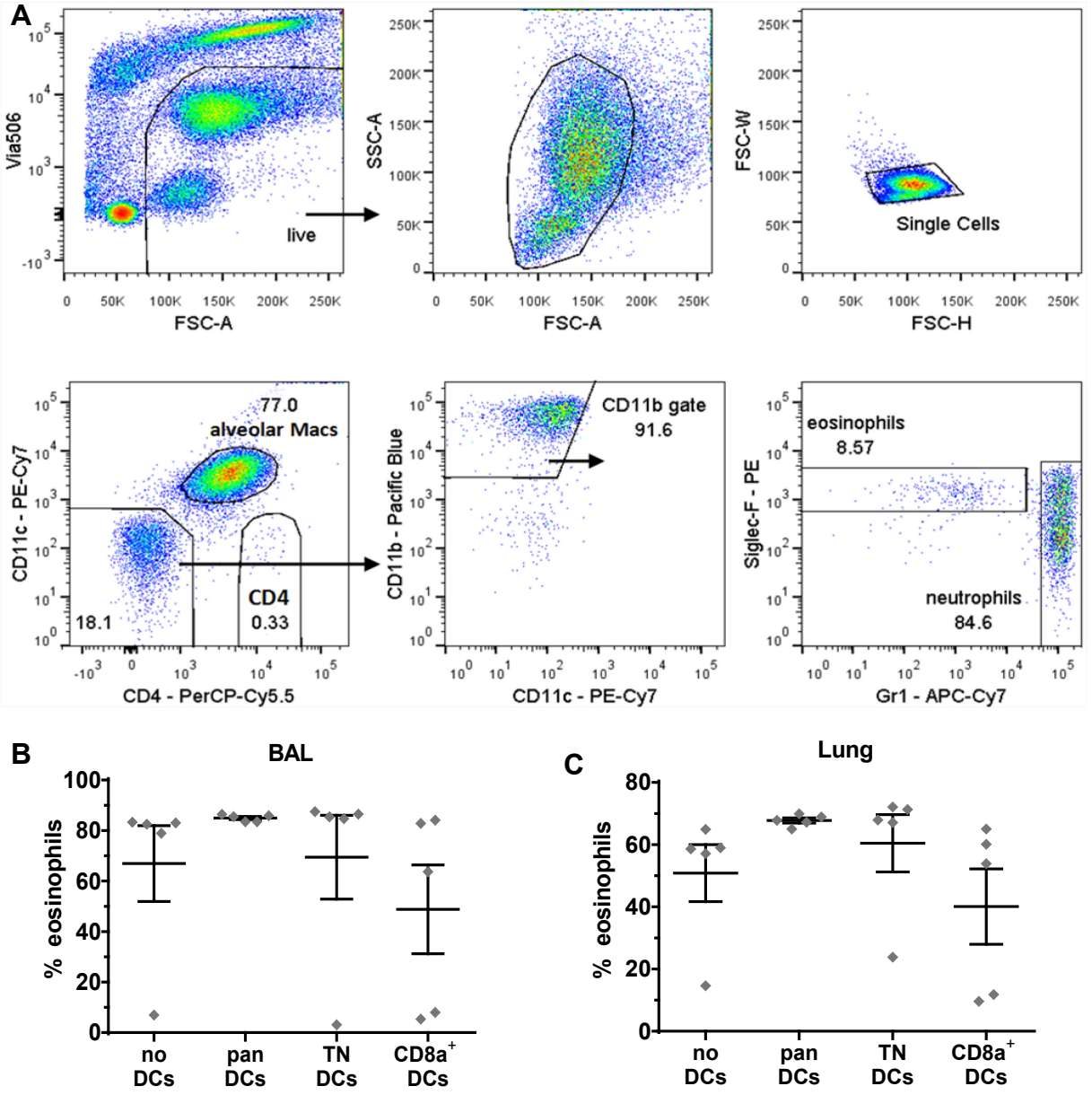

Fig. 30 Lungs from all mice (OVA sensitized at day 0, day 14 and OVA aerosol challenged for four days) were lavaged to collect bronchoalveolar fluid. Lungs were perfused, and lung tissue was digested to make single-cell suspension to collect eosinophils. CD8a$^+$ DCs (n=5), triple negative DCs (n=5), panDCs (n=5), or no DCs (control group, n=5) were administered intra nasal (i.n.) at a concentration of 5x10^5 / mouse. **(A)** FACS gating strategy for BAL fluid (example from CD8a$^+$ it-DC group with low eosinophil count). Alveolar macrophages are characterized as CD11c^{hi}, Siglec F$^+$, MHC II^{lo}, F4/80$^+$ in lung tissue and BAL. Eosinophils are CD11b$^+$ Gr1^{lo} Siglec F$^-$, whereas neutrophils are Siglec-F$^+$ Gr1^{hi}. Depicted numbers in gates are in %. **(B)** Numbers of eosinophils (Eos) were analyzed from BAL by cytospin analysis and **(C)** lung tissue. BAL fluid and lung tissue from CD8a$^+$ treated mice revealed that CD8a$^+$ might be able to induce tolerance with the lowest eosinophils count on average (mean; 48.8 % and 40.1%, respectively) compared to all other groups. 3 out of 5 mice in the CD8a$^+$ group showed a decrease in eosinophil numbers in BAL and in lung tissue, TN DCs in one mouse and pan DCs showed no decrease. Plots are depicted with mean ± SD.

Here, we induced an acute asthma-like reaction by systemic sensitization and challenge with aerosolized ovalbumin in mice (see methods). An imbalance of immunological tolerance is thought to be a main cause of the allergic asthma phenotype, and through the induction of ovalbumin specific Tregs by ovalbumin pulsed it-DCs, we expected to observe a decline in the severity of asthma-like symptoms and clinical determents of disease such as eosinophilia. In previous *in vitro* experiments, triple negative DCs (TN DCs), and $CD8a^+$ DCs drive Treg induction to a greater extent than other splenic DC subsets and thus were the most promising candidates.

Consequently, $CD11c^+$ pan it-DCs, TN it-DCs, and $CD8a^+$ it-DCs we administered intra nasal (i.n.) prior to antigen challenge with aerosolized ovalbumin. As an additional negative control, one group of mice did not receive DCs (no-DC group). Mice were sacrificed 24h after the last antigen exposure via inhalation. To determine eosinophilia, lungs from all mice were lavaged for BAL analysis and lung tissue was digested to determine eosinophilia as an indicator of airway inflammation.

$CD8a^+$ it-DCs had the lowest eosinophil count in BAL fluid and lung tissue (Fig. 30B and C). Surprisingly, there was no meaningful reduction of eosinophils in the TN group. An *in vitro* quality control of the administered it-DCs, untreated control DCs, and a positive control (ctrl-DC + TGFb/IL-2) are similar to our findings in the *in vivo* mouse model. $CD8a^+$ it-DCs demonstrated the highest Treg induction (46.1%), followed by TN it-DCs (24.4%), though TN exceeds $CD8a^+$ DCs in total cell numbers. This suggest that the induction was more Treg specific in $CD8a^+$ than in TN it-DCs (Fig. 29). *In vitro* Treg induction in TN DCs also had a lower percentage of $Foxp3^+$ Tregs than in previous experiments (36.9 ± 5.9%). Our results suggest that the administration of it-DCs as a potential therapeutic strategy to battle allergic asthma is a promising approach. However, further experiments need to confirm our most recent findings.

3.3 Generation of human induced-tolerogenic DCs

In our studies, we also investigated human DCs and monocyte-derived DCs from blood. To study these subsets we used a combination of several purification steps, magnetic labeling, and FACS sorting (see methods). We studied the following four DC subsets in our experiments: CD141 ($CD11c^+$ $HLA\text{-}DR^+$ $Lineage^-$ $CD141^+$), CD16 ($CD11c^+HLA\text{-}DR^+$ $Lineage^+$ $CD16^+$), CD1c ($CD11c^+$ $HLA\text{-}DR^+$ $Lineage^-$ $CD1c^+$), and pDCs ($CD11c^-$ $HLA\text{-}DR^+$ $Lineage^-$ $BDCA4^+$). Moreover, monocytes were isolated from blood and treated with GM-CSF and IL-4 for 6 days to obtain $CD11c^+$ monocyte-derived DCs (moDCs). All circulating blood-borne DCs and moDCs were cocultured with naïve human T cells from the same blood donor (autologous cells) for 5.5 days. T cells were cocultured with DCs and supplemented with anti-CD3 in four conditions: control DCs + T cells, LPS treated DCs + T cells, rapamycin and TGFb treated DCs + T cells, and control DCs + IL-2 and TGFb as positive control.

Experiments were difficult to evaluate due to low numbers of total viable cells (Via506 staining) *in vitro* in the cultures. However, in the last experiments we were able to gain more sophisticated data through the addition of human serum (same blood donor) to our cultures. Human serum caused to a boost in nearly all subsets in cell expansion and viability of cells in culture. This allowed us to identify discrete populations of $Foxp3^+$ $CD25^{hi}$ Tregs in moDCs and $CD16^+$ DCs.

3.3.1 DC subsets

3.3.1.1 DC distribution in blood

In four independent experiments, we determined the percentages of the four study DC subsets in human blood. The vast majority of human blood DCs are $CD16^+$ DCs, followed by $CD1c^+$, pDCs, and the rarest subset $CD141^+$ with only 4.2% on average (Fig. 31).

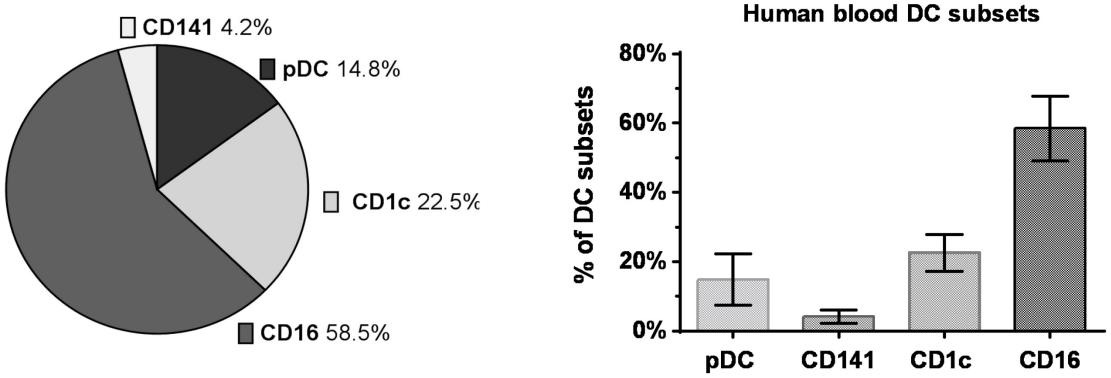

Fig. 31 Human blood DC subsets are depicted in percentages of total isolated DCs from four independent experiments (n = 4) with human peripheral blood from healthy male donors. Percentages are shown with mean ± SD. pDC (14.8 ± 7.4), CD141 (4.2±1.9), CD1c (22.5±5.4), and CD16 (58.5±9.3). $CD141^+$ DCs are the rarest subsets in human blood, followed by pDCs, $CD1c^+$ and $CD16^+$. Our results are in accordance to previous studies. {MacDonald 2002 #10}

3.3.1.2 Effect of human DC subsets on T cell responses in vitro

In the study of human Tregs, there is the lack of a definitive Treg cell markers. Contrary to murine cultures with naïve T cells, in humans Tregs are difficult to identify, since the canonical marker Foxp3 is also upregulated on activated T cells. The majority of T cells in our stainings show increased $Foxp3^+$ staining pattern that makes it difficult to identify populations with our three common markers in mouse (CD4, CD25, Foxp3). Other markers, such as CD39, CD45RA, CD45RO, and/or CD127 are used to identify Tregs. (Borsellino *et al.*, 2007) Tregs are often considered as $CD4^+$ $CD25^{hi}$ $CD127^{lo}$ $CD45RO^+$ $CD45RO^+$ $CD45RA^-$ $Foxp3^+$. (Duhen *et al.*, 2012).Thus in addition to examining Foxp3 expressing $CD4^+$ T cells, we also analyzed our experiments with CD45RO, and CD45RA, combined with CD25 as additional markers to identify T cell activation (or lack thereof) of the different subsets. All subsets were also analyzed by using CD127, but no meaningful observation was made. Cultures were harvested at day 5.

CD141

$CD141^+$ DCs resemble murine $CD8a^+$ DCs and play a role in self-tolerance. (Crozat et al. 2010) Studies demonstrated the capacity of $CD141^+$ DC to cross-present and produce IL-12p70, two functions in which mouse spleen $CD8a^+$ DCs excel. (Robbins et al. 2008; Kushwah and Hu 2011) Accordingly, considering our successful murine studies with $CD8a^+$ DCs, $CD141^+$ DC was a promising candidate in terms of T cell activation. Analyzing $Foxp3^+$ induction was inconclusive. Therefore, T cell activation was analyzed by CD45RO and CD25 expression. Despite similarities between this mouse and human subset, we were unable to identify $CD45RO^+$ $CD25^+$ T cell activation in $CD141^+$ it-DCs. Contrary, we observed that CD141 it-DCs seem to be unable to activate naïve T cells to $CD45RO^+$ $CD25^+$ $CD4^+$ at all and $CD4^+$ cells appeared locked in a more naïve state $CD45RO^-$ $CD25^-$ (Fig. 32A). However, control DCs and positive control were able to activate naïve T cells to some extent (14% and 16.1% of all $CD4^+$ T cells). Interestingly, we observed in three independent experiments, regardless of culture media, that total CD25 and CD45RO expression (staining pattern) on $CD4^+$ cells is lower compared to T cells stimulated by other human blood DC subsets. Due to low yield of $CD141^+$ DCs gained from blood, testing with AIM-V + human serum was not conducted. Nonetheless, we do not expect to see a meaningful difference in T cell activation by taking previous results into consideration.

CD1c

$CD1c^+$ DCs may be functional equivalent to murine $CD11b^+$ cDCs. (Crozat *et al.*, 2010) However, instead of elevated T cell activation, we largely observed the opposite in $CD1c^+$ DCs. Though statistical significance between the control group and it-DCs has not been

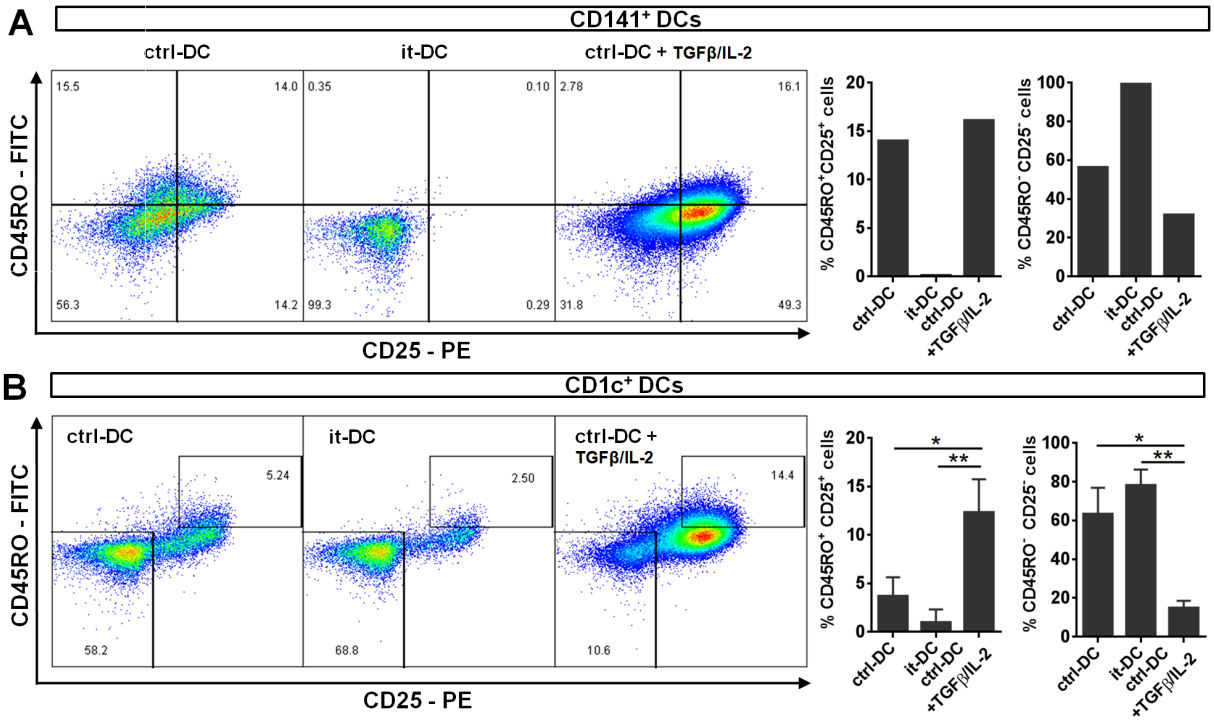

Fig. 32 (A) CD141 coculture with naïve T cells (AIM-V medium) at day 5. In this and previous experiments with other media we were unable to see any CD45RO$^+$CD25$^+$ T cell activation by CD141$^+$ it-DCs (data not shown due to minor experimental alterations). CD141$^+$ it-DC group showed inferior cell viability (57.7%) compared to ctrl-DCs (79.8%) and ctrl-DCs+TGFb/IL-2 (82,6%). CD141$^+$ it-DCs are unable to induce CD45RO$^+$ CD25$^+$ CD4$^+$ T cells (0.1%) and CD4$^+$ cells are locked in CD45RO$^-$ CD25$^-$ (99.3%; see right figure). **(B)** CD1c$^+$ DC gating on CD45RO$^+$ CD25$^+$ and CD45RO$^-$ CD25$^-$cells. Cells were pre-gated for viability with Via506 and CD4. Differences between CD1c$^+$ it-DC, ctrl-DC and the positive control group (ctrl-DC+TGFb/IL-2) are statistically significant. Though statistically significance between the control group and it-DCs has not been reached due to a low number of experiments (n=3), the it-DC group may cause a development delay or inhibition that locks T cells in a more naïve state compared to ctrl-DCs and positive control. one-way ANOVA with Tukey's multiple comparison test was used; n=3, p = 0.0031)

reached due to a low number of experiments (n=3), the it-DC group may cause a development delay or inhibition that locks T cells in a more naïve state compared to ctrl-DCs and positive control. The positive control group is capable of inducing Foxp3$^+$ CD25$^+$ Tregs that can be clearly identified as a discrete subpopulation (1% of CD4$^+$ CD45RO$^+$ CD25^{hi}) of CD4$^+$ CD45RO$^+$CD25^{hi} Foxp3^{hi} cells (data not shown). The left plot in Fig. 32B depicts the vast difference in percentages of total viable CD4$^+$ CD45RO$^+$ CD25$^+$ cells between the it-DC group (mean±SD; 0.95±1.35)) and the positive control (12.3±3.4). However, even the ctrl-DC group (3.6±1.95) demonstrates higher T cell activation capability (3.6 ±1.95) compared to CD1c it-DCs. CD1c it-DCs (78.1±8.2) have the highest percentage of CD4$^+$ CD45RO$^-$ CD25$^-$ ctrl-DCs compared with ctrl-DCs (63.3±13.6) and ctrl-DCs+TGFb/IL-2 (14.8±3.7). The graph (right) in Fig. 32B reinforces that CD1c$^+$ it-DCs are rather inhibiting T cell activation than promoting it. Moreover, culture conditions are at a level that allow sufficient cell growth and expansion of cells in these cultures, resulting in cultures with a high percentage of viable cells (data not shown).

CD16

In $CD1c^+$ DC cultures no improvements were observed in cell viability, cell numbers, or T cell activation through the addition of human serum to cultures. Contrary, AIM-V + human serum lead to a peak in cell growth for $CD16^+$ DC cocultures with viability rates that increased by up to 25% compared to previous cultures without HS (Fig. 33A lower right diagram; ctrl-DC 90%, it-DC 96%, and positive ctrl 97.5% of all cells in culture). Total cell numbers also increased dramatically in all groups (ctrl-DCs (27x), it-DC (14x), and positive ctrl group (29x)). The it-DC group also reveals a huge increase in numbers of $CD45RO^+$ $CD25^{hi}$ activated T cells (36.6% of pre-gated viable $CD4^+$ T cells) compared to the ctrl-DC (10.1%) and positive ctrl group (8.39%). Likewise, naïve $CD4^+$ $CD45RO^-$ $CD25^-$ T cells are lowest in the it-DC group (29.2%), followed by positive ctrl (39.3%), and ctrl group (51.2%). Interestingly, the it-DC group may activate T cells in a more specific way compared to ctrl-DCs and ctrl-DCs + TGFb and IL-2. It is the only group where subpopulations can be identified in two quadrants. Whereas the other groups reveal a lower and more unspecific activation (lower CD25 expression) that seem to lead to a more general unspecific activation of T cells resulting in the depicted FACS plot pattern.

Additionally, we clearly identified a small but distinct population of $Foxp3^+$ $CD4^+$ $CD25^{hi}$ Tregs induced by $CD16^+$ it-DCs (0.63% of $CD4^+$ viable cells). Though, the ctrl-DCs + TGFb and IL-2 also induced $Foxp3^+$ $CD25^{hi}$ Tregs (0.34%), induction was inferior in percentages and in total numbers of Tregs. $CD16^+$ DCs excel in cell viability, proliferation, T cell activation, and $Foxp3^+$ $CD25^{hi}$ Treg induction over all other human blood DC subsets in our studies.

pDC

Neither untreated control DCs nor rapamycin and TGFb treated pDCs demonstrated any capacity to activate T cells. Even overstimulation with IL-2 and TGFb in the positive control was not sufficient to elicit a meaningful $CD45RO^+$ $CD25^+$ T cell activation (2.43%) in pDCs. The huge majority of all cells are still in a naïve state $CD45RO^-$ $CD25^-$; ctrl-group (mean; 99.1%), it-DC (98.9%) and ctrl-DC + TGFb and IL-2 (71.6%). These results were consistent in three independent experiments.

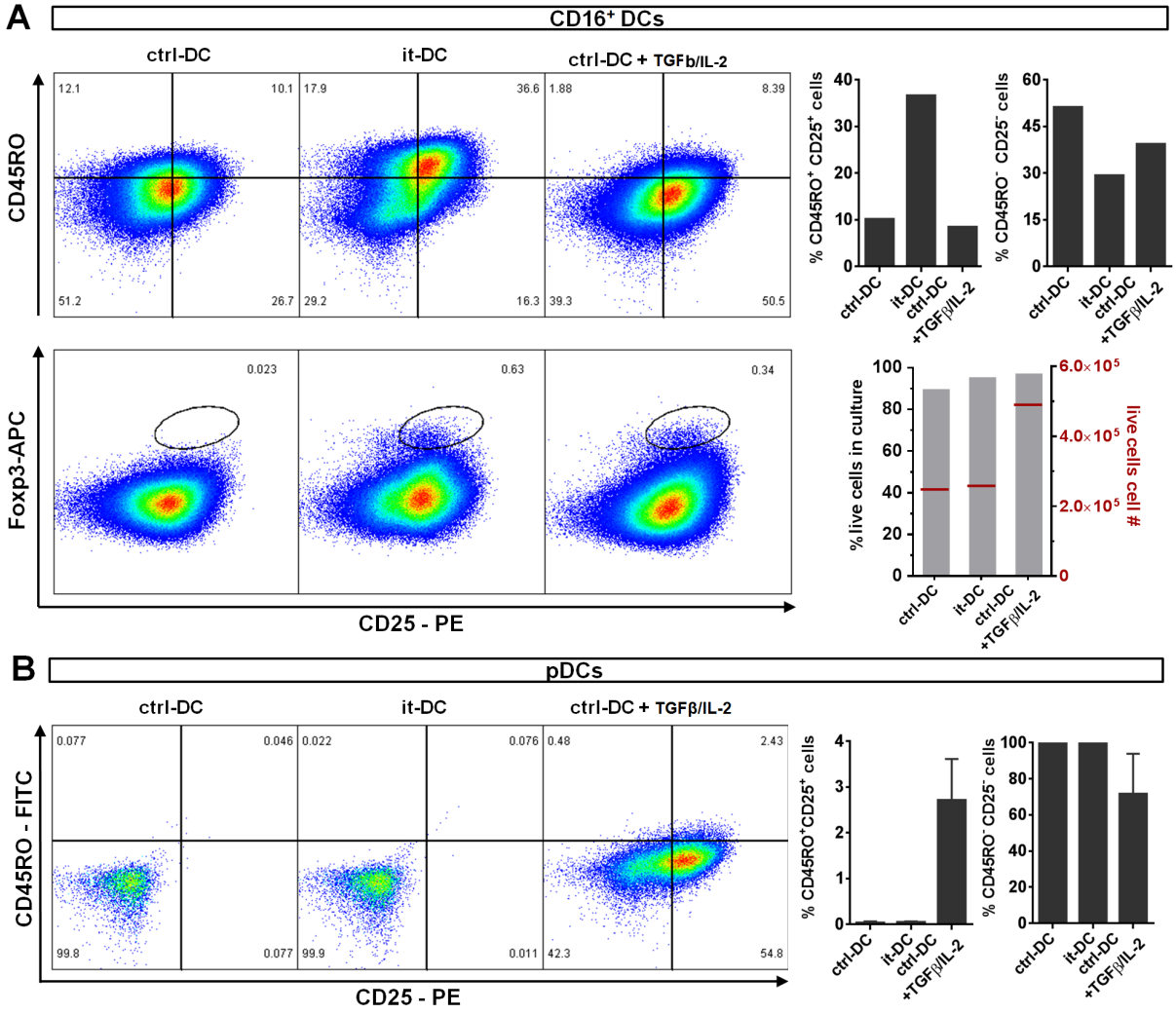

Fig. 33 (A) CD16$^+$ DCs were analyzed using CD45RO and Foxp3-APC and cells were cultured in AIM + HS. The it-DC group showed remarkable T cell activation and Foxp3 induction that was superior to all other groups. **(B)** pDCs were gated with CD45RO. Rapamycin treatment did not lead to any T cell activation in regard to CD25 or CD45RO expression in three independent experiments. Addition of IL-2 and TGFb in the positive control group stimulated T cell activation (CD45 RO expression), but still only low CD25 surface expression levels were detected. CD45RO$^-$ CD25$^-$ gates; ctrl-group (mean±SD; 99.1±0.5), it-DC (98.9±0.7) and ctrl-DC + TGFb/IL-2 (71.6±22.3). CD45RO$^+$ CD25$^+$ gates in the ctrl (0.03±0.03), it-DC (0.4±0.02), and ctrl-DC + TGFb/IL-2 group (2.7±0.9).

3.3.2 monocyte-derived DCs

Here, we were able to clearly show the effect of it-DCs on T cells with our optimized culture conditions. In previous experiments, T cell activation occurred in a manner that obscured the differentiation between Foxp3$^+$ Tregs and other potentially Foxp3 expressing activated T cells (Fig. 34A). Culturing solely in AIM-V without human serum lead to an unspecific increased T cell activation of all CD4$^+$ cells along with increased Foxp3 expression. We did not include data from previous experiments, because different gating strategies may lead to biased results and it is still a controversial issue as to what markers truly define a Treg in humans. In contrast to mouse, it is difficult to identify human Tregs solely by their expression of Foxp3$^+$ CD25^{hi}, because other T cells also express both markers, but do not necessarily share other Treg features.

Our experiment with different culture conditions (Aim-V, AIM-V+HS, AIM-V+HS+IL2) revealed that it-DCs have a substantial impact on T cell differentiation. Though it seems in Fig. 34A that all DC groups are capable to activate CD4$^+$ T cells likewise, this observation may not be true when compared to other cultural conditions where human serum (and IL-2) was added (Fig. 34B and C).

First, the addition of human serum lead to an increase in percentage of total viable cells in culture in all groups except it-DCs. However, the it-DC percentage of viable cells was already much higher with AIM-V compared to ctrl-DCs and LPS-DCs. Due to experimental variations, it-DCs cultured in AIM-V+HS showed a low total cell number what might also have caused a bias in percentages of viable cells. There is a huge variety of different cytokines in human serum and among those are also IL-2 and TGFb. Though an excessive amount of IL-2 and TGFb was added to the positive group, percentages in total viable cells are still increasing. This suggests that IL-2 and TGFb in human serum does not significantly contribute to increased cell viability.

Second, optimized culture conditions with human serum did not only lead to "healthier" cultures but as a consequence of that we were able to observe the effects of the different DC groups (Fig. 34B). This might due to the addition of human serum that resulted in a more physiological response compared to the generalized unspecific T cell activation of all T cells. Control and LPS-DCs gave rise to two distinct populations (CD45RO$^-$ CD25$^-$ and CD45RO$^+$ CD25$^+$), whereby LPS-DCs show a slightly stronger pronounced CD45RO$^+$ CD25$^+$ population than expected. However, it-DCs showed inhibition of T cell activation and the majority of CD4$^+$ T cells appeared "locked" in a naïve state (CD45RO$^-$ CD25$^-$), though a small population was already CD45RO$^+$ CD25$^+$. The positive control (ctrl-DCs + TGFb and IL-2) shows an unspecific generalized T cell activation where also no distinct Foxp3$^+$ CD25$^+$ CD45RO$^+$ subpopulations could be detected (data not shown).

Third, culturing media with AIM-V containing human serum and IL-2 leads to an optimal stimulated T cell culture with maximum percentage of viable cells. Control-DCs and LPS-DCs show similar T cell activation, but not it-DCs. Interestingly, it-DCs show a more specific T cell

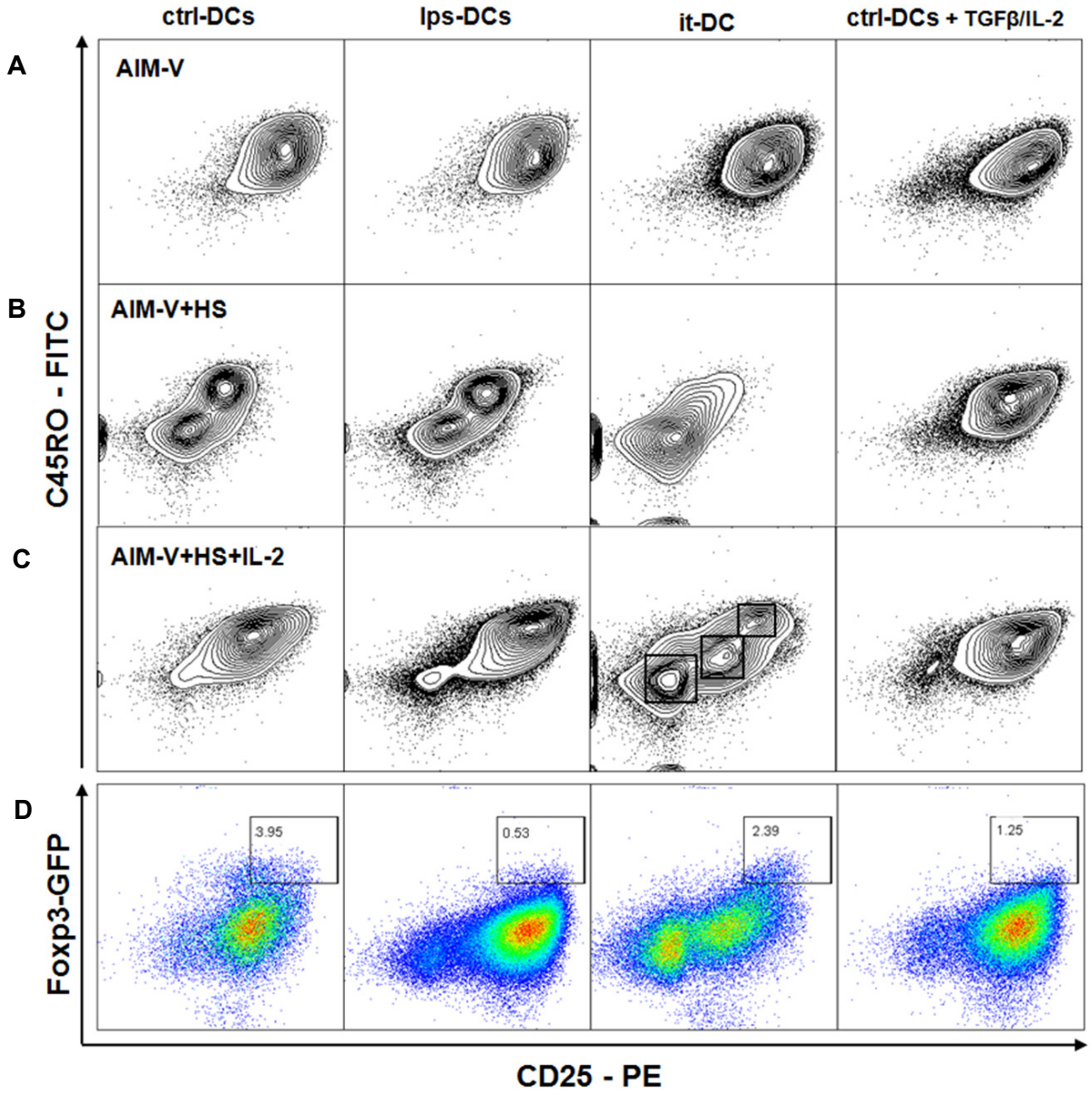

Fig. 34 Culture condition optimization lead to an increase in percentages of viable cells (gated with Via506) and demonstrated specific it-moDC T cell activation capability. Three different culture conditions (AIM-V medium **(A)**, AIM-V + human serum (HS) **(B)**, AIM-V medium with HS and Interleukin-2 (IL-2; 200 U/ml) were assessed in one experiment **(C)**. T cells and moDCs were used from the same donor in all cultures. ctrl-DCs, LPS treated DCs, rapamycin + TGFb treated moDCs (it-DCs), and ctrl-DCs with added TGFb and IL-2 were compared in regard to surface expression of CD45RO and CD25; both markers indicate T cell activation. **(D)** Gating on $Foxp3^{+}$and $CD25^{hi}$ revealed a small distinct population of Tregs (2.39%) in the it-DC group. Though the ctrl-DC+TGFb/IL-2 group (1.25%), LPS-DC group (0.53%) as well as ctrl-DCs (3.95%) show an induction of $Foxp3^{+}$ $CD25^{hi}$, it needs to be noted that there are no distinct subpopulations. ctrl-DCs rather show an unspecific activation with a wider range of CD25 expression rather than a more typical $CD25^{hi}$ population that can be distinguished from other $CD25^{+}$ T cells. The total number of $Foxp3^{+}$ $CD25^{hi}$ cells is also higher in the it-DC group (Fig. 36).

activation with three clearly defined populations that may represent different developmental stages.

it-DCs showed also for the first time a small, but distinct population of Foxp3^{+} CD25^{hi} population (Fig. 34D). Though the positive control always showed remarkable T cell activation, we were not able to detect a subpopulation that we would classify as Foxp3^{+} CD25^{hi} Tregs. Notably, ctrl-DCs showed a population of Foxp3^{+} CD25^{hi} cells, but compared to it-DCs, it is an inhomogeneous population with a wide variability of CD25 surface markers.

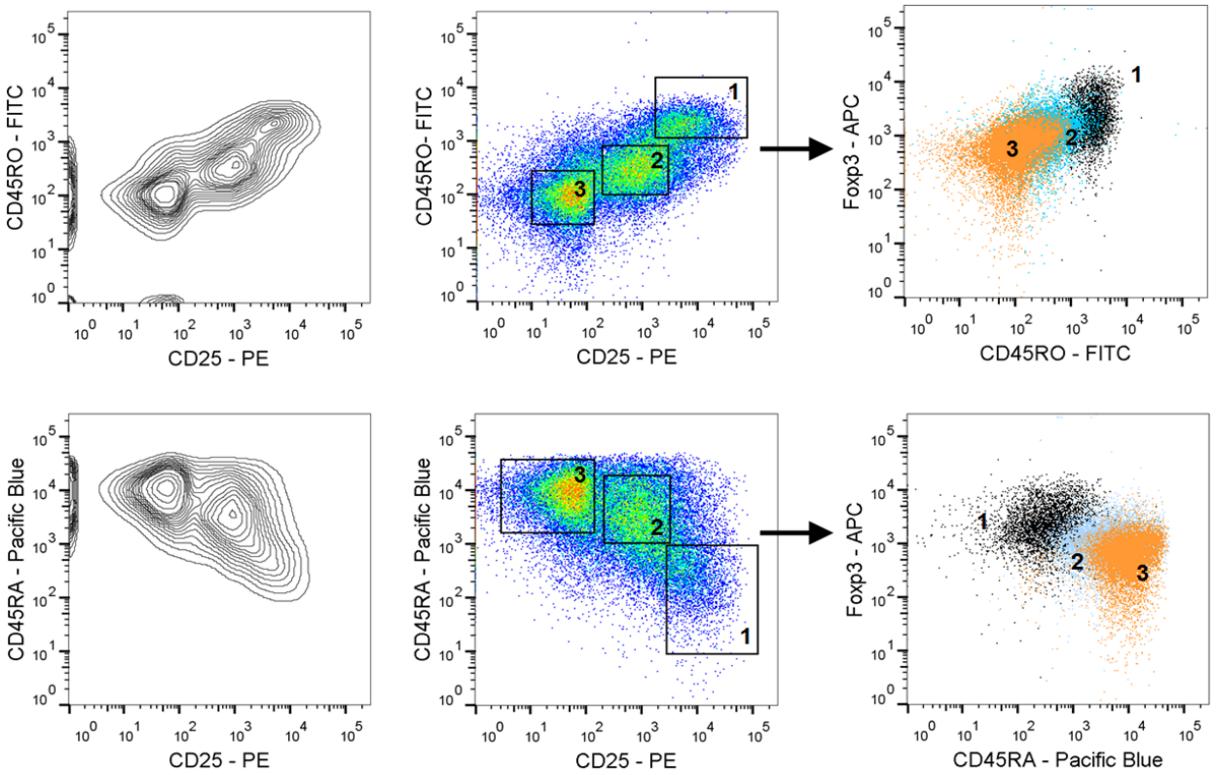

Fig. 35 To further examine the three subpopulations of the it-DC group and confirm that those resemble different developmental stages, a direct comparison in regard to Foxp3 expression and CD45RA or CD45RO was done. Discriminated by color, CD45RO^{+} CD25^{hi} T cells (group 1 in black) are expressing more Foxp3 than CD45RO^{int} CD25^{int} (group 2 in light blue) and CD45RO^{-} CD25^{-} (group 3 in orange) T cells. Likewise, CD45RA^{+} CD25^{-} (immature T cells, group 3 in orange) show the lowest Foxp3 expression. With the decline of CD45RA on the surface, Foxp3 expression increases and is highest in CD45RA^{-} CD25^{+} cells (group 1 in black). Depicted are CD45RO and CD45RA on the y-axis and CD25 on the x-axis in two different diagrams. CD45RO and CD25$^{+/hi}$ are markers of activated Tregs, whereas CD45RA^{+} cells are in a more naïve state. In the upper row: The gating revealed that CD45RO^{hi} CD25^{hi} cells (gate 1) show on average higher Foxp3^{+} expression compared to CD45RO^{int} CD25^{int} (gate 2) and CD45RO^{-} CD25^{-} (gate 3). Likewise, it can be observed in the lower row that the third gated population in the upper row overlaps with its characteristics with the population of gate 3 in the lower row. The population in gate 3 is CD25^{-} CD45RA^{+} and Foxp3$^{-/lo}$. Likewise this can be observed with the other gates as well (see accordingly colored population in the last gate).

In conclusion, cultures with AIM-V show a major capability to activate T cells, but in an unspecific way that was little influenced by treatment with LPS, rapamycin and TGFb, or the addition of TGFb and IL-2. Therefore, no major differences could be detected by gating on CD45RO (or Foxp3 (data not shown)) and CD25. However, the addition of human serum lead to significantly higher percentages of viable cells, but also to a more specific activation

of T cells. For the first time, we were able to see meaningful differences by identifying subpopulations in ctrl-DCs, LPS-DCs, and it-DCs (Fig. 34B and C). Rapamycin is known to cause a developmental delay in T cells as suggested in the it-DC AIM-V+HS culture. The addition of IL-2 to all cultures lead to shift towards $CD45RO^+$ $CD25^+$ T cells with ctrl and LPS-DCs. However, the it-DC group showed a pattern that clearly depicts three subpopulations in different developmental stages.

Here, we suggest that monocyte derived it-DCs inhibit the generation of $CD45RO^+$ $CD25^+$ T cells from $CD45RA^+$ naïve T cells. Moreover, monocyte-derived it-DCs are capable of specifically inducing $Foxp3^+$ $CD25^{hi}$ Tregs. Furthermore, moDCs have a greater potential to induce T cell activation compared to the other investigated blood-borne human DC subsets.

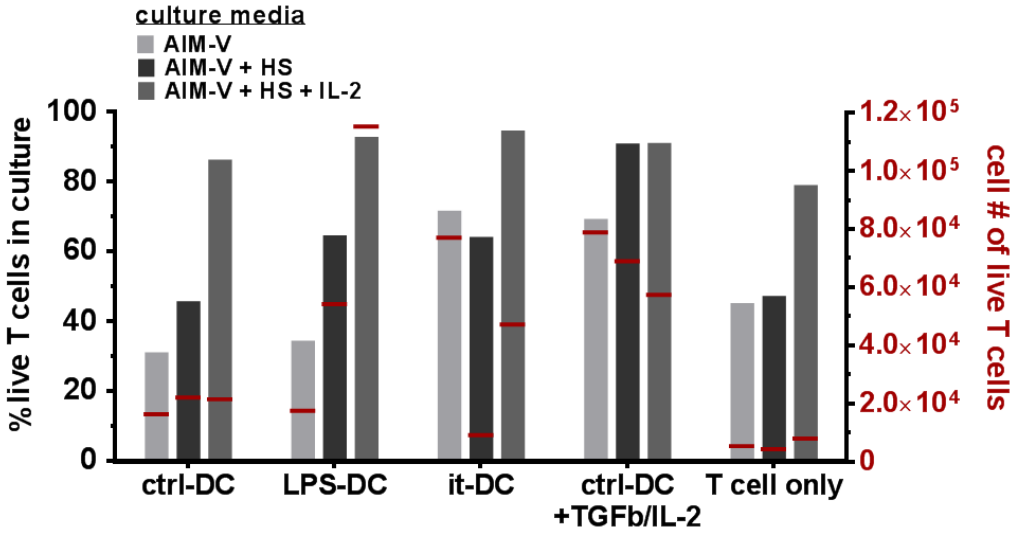

Fig. 36 Cell viability (in percentages and total cell numbers) are depicted for moDCs/naïve T cell co-cultures, but also monocultures of naïve T cells (T cell only) in different culture conditions (AIM-V, AIM-V+HS, AIM-V+HS+IL-2). Cocultures with ctrl-DCs, LPS-DCs, it-DCs, and ctrl-DC with TGFb/IL-2 are shown. It can be seen that percentages of live T cells (left y axis) raises in all groups with AIM-V+HS+IL-2. It can be observed that IL-2 itself has an effect on T cell viability (see T cell only), but it is minor when total cell numbers are taken into consideration. However, in the ctrl-DC, LPS-DC, and it-DC group viability increases of up to 94.9% (it-DCs) what is a remarkable height and was not achieved in any other experiment with DCs either in human nor in mouse. It can be seen that all DC groups peak in cell viability in AIM-V+HS+IL-2. The ctrl+TGFb/IL-2 group already does not further raise in percentage, because IL-2 is already in the culture medium and an excess does not have any further effect on cell viability. Total cell numbers of live T cells (right y axis) are shown, but can't be considered for evaluations because of variability between experiments is common and needed to be repeated. However, T cell only cell numbers are consistently very low and all in a naïve state ($CD4^+$ $CD25^-$ $CD45RO^-$ $CD45RA^+$; data not shown) and are representative. Additionally, it-DCs cultured in AIM-V+HS show a low total cell number what might have also affected the outcome of percentages of viable cells.

3.4 Live cell imaging

Next, we examined human blood DC subsets (CD1c, CD16, CD141 and pDCs) as well as murine DCs, by *in vitro* videomicroscopy in order to understand some of the morphological and behavioral differences of these subsets at steady state. Humans DCs were incubated without other cells to evaluate morphology and morphological changes with conventional light microscope video imaging over the course of time (up to 72h). The high purity of the subsets allowed us to clearly identify some of the phenotypes of the different subsets. In the mouse, we added naïve $CD4^+$ T cells (from Foxp3-GFP reporter) to visualize the *de novo* induction of Foxp3-GFP^+ Tregs.

The following videos are digitally attached to this thesis and internet inks are provided below. Screenshots of movies 1 to 7 can also be found in "Attachments".

- movie 1: pDC monoculture (80x magnification, time lapse: 48h, video length: 60 sec). http://tiny.cc/human1
- movie 2: $CD141^+$ DC monoculture (40x magnification, time lapse: 48h, video length: 62 sec). http://tiny.cc/human2
- movie 3: $CD16^+$ DC monoculture (40x magnification, time lapse: 48h, video length: 60 sec). http://tiny.cc/human3
- movie 4: $CD1c^+$ DC monoculture (40x magnification, time lapse: 48h, video length: 62 sec). http://tiny.cc/human4

All imaged DCs were pulsed with ovalbumin and stimulated with rapamycin and TGFb according to protocol. T cells were naïve OT-II Foxp3-GFP negative $CD4^+$ T cells. In the following three videos, the $CD4^+$ T cells were sorted as naïve; therefore at the start of culture they are negative for Foxp3, and consequently not GFP^+.

- movie 5: $CD8a^+$ DC / T cell coculture (40x magnification time lapse: 48h, video length: 60sec). http://tiny.cc/murine1
- movie 6: TN DC / T cell coculture (40x magnification time lapse: 48h, video length: 60sec). http://tiny.cc/murine2
- movie 7: $CD11b^+$ DC / T cell coculture (40x magnification time lapse: 48h, video length: 60sec). http://tiny.cc/murine3
- movie 8: $Foxp3^+$ Treg cell division (40x magnification, video length: 9 sec). http://tiny.cc/murinefoxpd
- movie 9: Dendritic cell and $Foxp3^+$ Treg cluster (40x magnification, video length: 52sec). http://tiny.cc/murinefoxpc

3.4.1 Visualization of murine DC subsets

The following images of splenic DC subsets sorted from RFP reporter mice (DCs fluorescence in red channel) were cocultured with naïve $CD4^+$ T cells. $CD8a^+$ DCs have longer thin dendrites and an oval-shaped dense cell body. Within the first 24 hours, DCs develop to a round shaped cell body where round vacuole-like structures start to appear within individual cells and fill up the cytoplasm. After 48h, approximately 90% of all DCs attached together in huge DC clusters.

In contrast, the majority of $CD11b^+$ DCs seem to better attach to the surface and are therefore depicted with a flat cell body with shorter and wider dendrites compared to $CD8a^+$ DCs. Within the first 24h, $CD11b^+$ DCs detach from the surface and become more round shaped dense cell body with few short dendrites and start to gather within 24 hours in clusters of up to about 15 DCs. After 48 hours, all DCs are gathered into a few large clusters of over 100 DCs with only a few DCs outside the clusters in the culture.

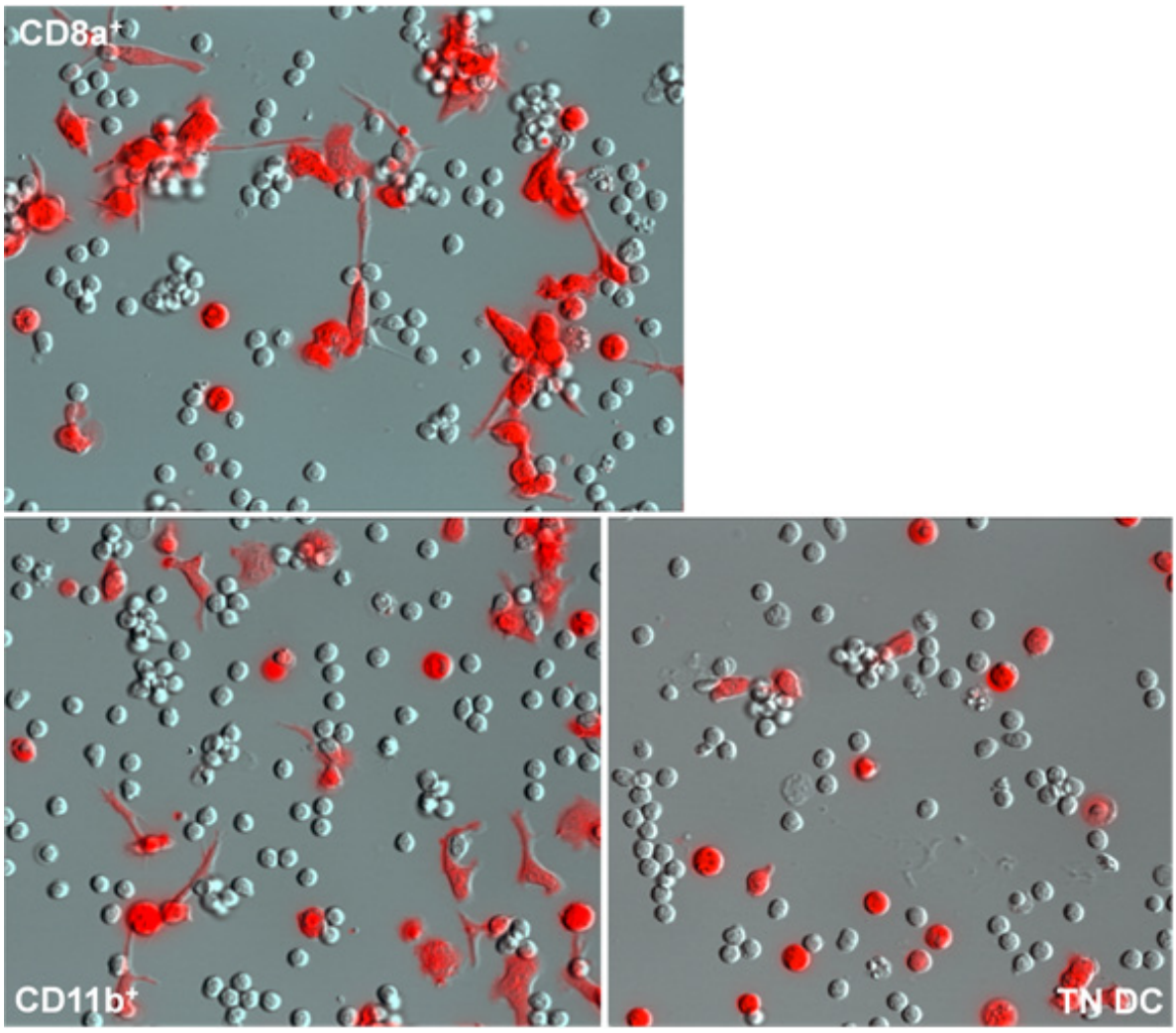

Fig. 37 Murine DC subsets $CD8a^+$, $CD11b^+$, and triple negative (TN) are cocultured with naïve $CD4^+$ T cells (40x magnification). DCs are red fluorescing and T cells are not fluorescing.

Triple negative DCs show a more diverse phenotype than $CD8a^+$ and $CD11b^+$ DCs, whereby some resemble $CD8a^+$ DCs in regard to their dendritic extensions, but the majority are rather small round-shaped cells, which are just slightly larger than the T cells. Over the course of time, TN DCs do not show a major morphological transformation, but some also develop round vacuole-like structures seen also in $CD8a^+$ DCs. TN DCs gather in clusters similar to those that were observed $CD11b^+$ DCs.

3.4.2 Visualization of human DC subsets

CD1c, CD16, CD141 and pDCs have all nearly the same cell size, but differ significantly in their cellular appearance.

$CD141^+$ DCs are round shaped cells that have few short individual dendrites. Though the purification of this subset followed the same protocol than the other subsets, debris of some kind were observed in culture. Cells do not show major morphological changes, but rather seem to undergo apoptosis with the larger part of the population being dead after 48h in culture.

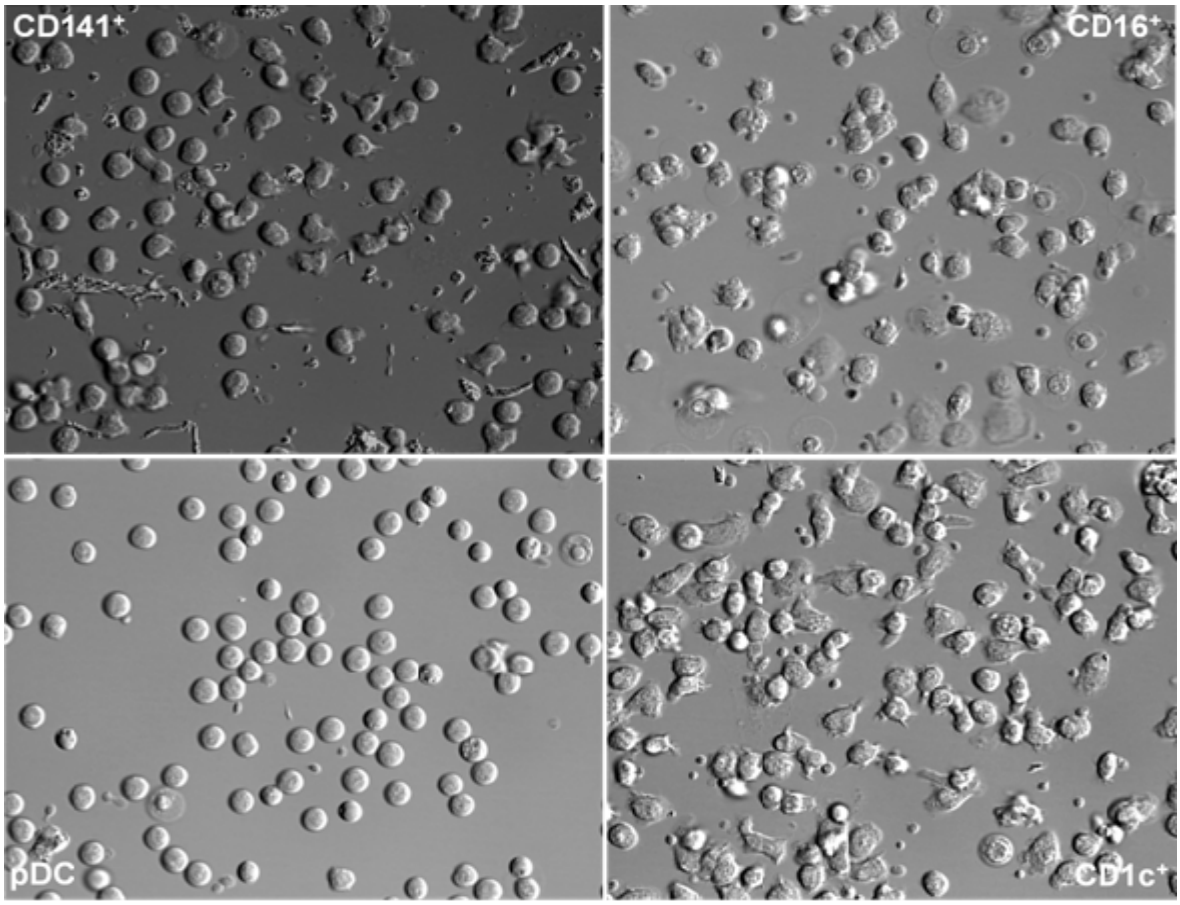

Fig. 38 Human DC subsets imagined immediately after the culture was incubated (time point 0h). Depicted are monocultures in TIMM media of $CD141^+$, $CD16^+$, $CD1c^+$ DCs, and pDC.

$CD16^+$ DCs are round shaped cell that have multiple tiny dendrites all over the cell surface. Within the first 36h, they massively extend their dendrites, attach to the surface, and build a huge network with their dendritic extensions where it seems that every viable cell is continuously in contact with at least one other dendritic cell through their dendrites. (Fig. 39). Remarkably, $CD16^+$ DCs are highly viable even after 96h in monoculture without the addition of cytokines or addition of fresh cell medium. (see Attachments)

pDC can be easily identified as nearly perfect round cells. Though, interactions between pDCs is observed, nearly all cells are dead within 48 hours with no morphological changes prior of undergoing apoptosis.

$CD1c^+$ DCs are more diverse in terms of cell shape, but can still be clearly distinguished from other DC subsets through their stretched oval to round shape with short dendrites that are slightly longer compared to those of $CD16^+$ DCs. Within 24 hours, the majority of $CD1c^+$ DCs gather in small clusters of about 20 DCs by keeping their initial cell shape. However, a few DCs attach closely to the surface that are depicted as long flat and wide stretched cell bodies that seem to explore the entire area by interacting with the groups of

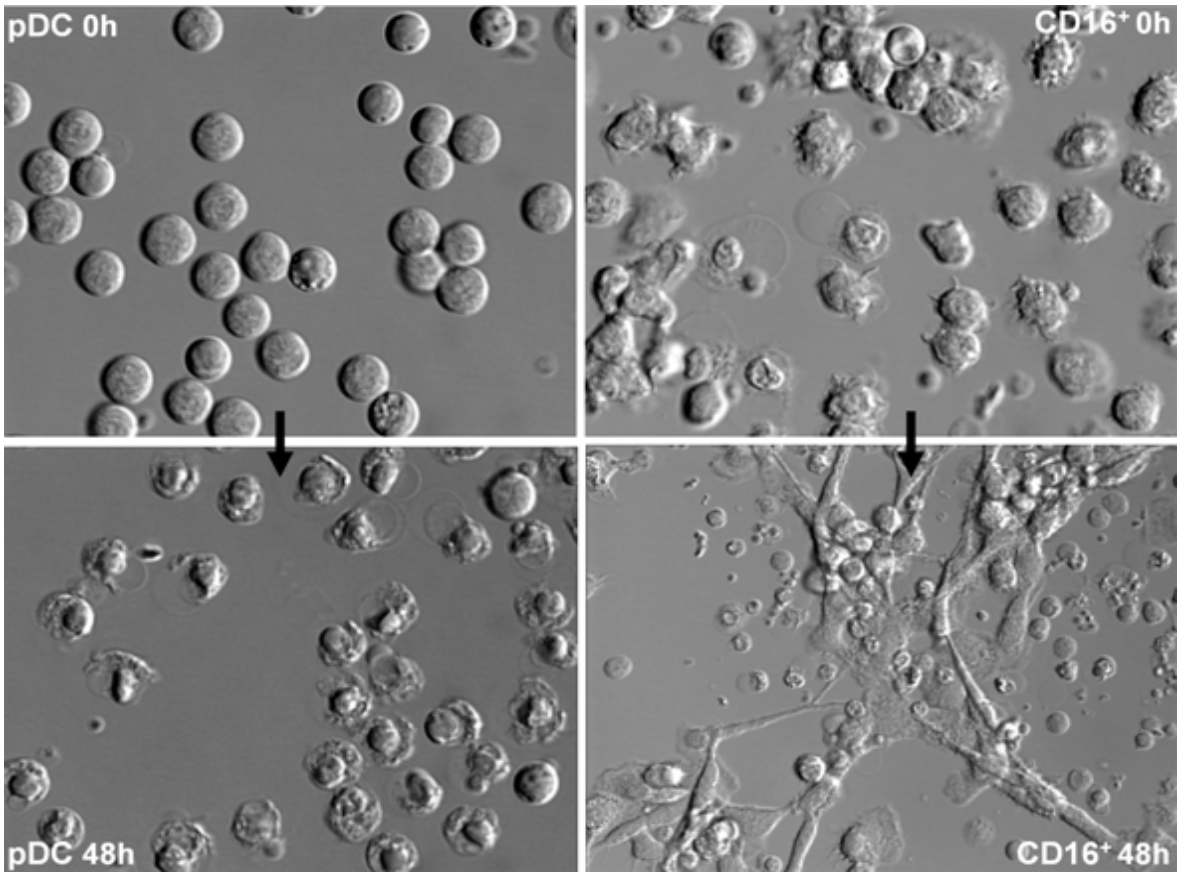

Fig. 39 pDC and $CD16^+$ DCs are depicted in 80x magnification immediately after the culture was incubated (0h) and 48 hours later. Whereas the vast majority of pDC is already apoptotic and not viable anymore at 48h after incubation, $CD16^+$ DCs make a transformation from their original form and build huge networks where all DCs are connected to each other and is fully viable at this point. Even after 96 hours, $CD16^+$ DCs are still alive and interact with each other through these dendritic networks, though no other stimulus was added to the culture. Both cell types have nearly the same size at 0h.

round shaped CD1c$^+$ DCs. Moreover, these two states are interchangeable by observing that only few CD1c$^+$ round shaped DCs transform into these flat stretched exploring CD1c$^+$ DCs. However, this transformation does not seem to be a developmental step, because, even after 96h in monoculture, only a minority of CD1c$^+$ are attached flat to the surface, whereby the rest of the cells kept their initial shape. Contrary, all CD16$^+$ DCs lose their initial round shape and create a huge dendritic cell network. Surprisingly, even after 96h in culture, similar to CD16$^+$ DCs, the bigger part of CD1c$^+$ is still viable.

3.4.3 Visualization of Foxp3$^+$ Treg proliferation

In addition to visualizing the interactions between DCs and naïve CD4$^+$ T cells, we also sought to image the generation of Tregs as well as their fate (post induction). For the first time reported, we observed by live cell imaging the division of a terminally differentiated Foxp3-GFP$^+$ CD4$^+$ T cell (Fig. 40). Green fluorescence indicates Foxp3 expression from the GFP reporter. Additionally, a video is attached that shows the cell division (movie 8). We have not found other publications that were able to show the division of differentiated Foxp3$^+$ regulatory T cells. This is suggestive that Tregs divide as Foxp3$^+$ cells under non inflammatory conditions and therefore directly inheriting the tolerogenic ability from the mother cell.

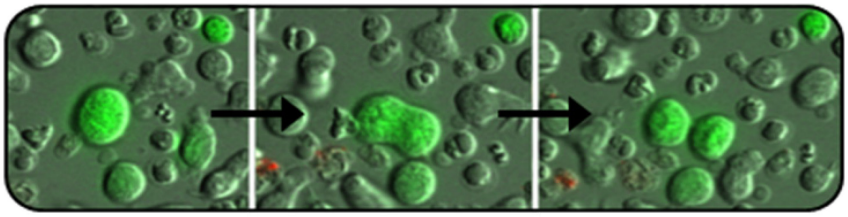

Fig. 40 Live cell video imaging depicts a single Foxp3$^+$ T cell (green in the middle). The cell divides within minutes into two separate Foxp3 expressing T cells. Surrounding non fluorescing cells are CD4$^+$ T cells that do not express Foxp3.

3.4.4 Visualization of DC clustering

In a CD11c$^+$ panDC culture, non fluorescing CD4$^+$ and green Foxp3$^+$ CD4$^+$ Tregs with red panDCs are depicted (it-DCs). Over the course of time, DCs gather and form huge clusters of DCs that are highly active and migrating in the culture as one single large cluster together. In these cultures, a variety of different Foxp3$^+$ expressing T cells can be observed. These Tregs differ in size and shape, whereby the larger nearly perfect round shaped Tregs were observed in active proliferation (Fig. 40). Moreover, it can be observed that already Foxp3 expressing T cells are migrating into these cluster. In the center of these large clusters, the green fluorescence outshines the red from the DCs and the core appears to be green and is encircled by red DCs.

Here, we suggest that after initial stimulation by DCs, T cells may transmit signals that makes further stimulation obsolete and DCs start gathering in clusters. Another reason for this DC clustering could be mutual cytokine stimulation. Additionally, T cells that already express Foxp3 are in a developmental stage where no further DC stimulation seem to be necessary for Treg proliferation. However, Foxp3 expressing Tregs also tend to form clusters together with DCs. If initial T cell activation also takes place in these clusters need to be further investigated.

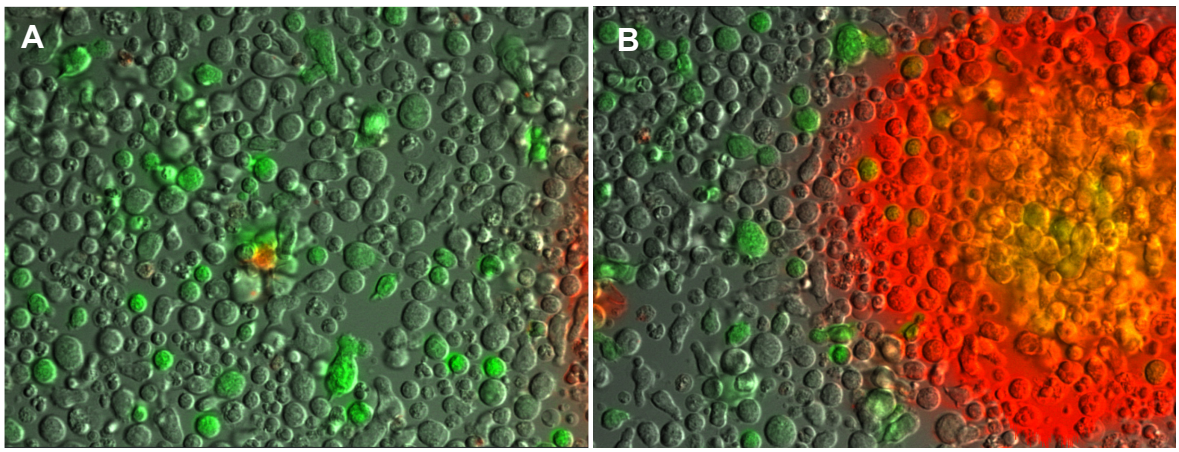

Fig. 41 $CD11c^+$ RFP DC and OT-IIxFoxp3-GFP $CD4^+$ T cell coculture at day 3. **(A)** Individual green $Foxp3^+$ expressing T cells with different sizes and shapes are depicted. **(B)** Large cluster of DCs that appear to surround Tregs (see movie 9).

4 Discussion

4.1 Murine *in vitro* and *in vivo* experiments

In our studies, we showed that rapamycin and TGFb treatment of $CD11c^+$ dendritic cells from the spleen displayed a significant increase in the ability to induce antigen-specific $Foxp3^+$ $CD25^+$ regulatory T cells from naïve T cells compared to naïve splenic DCs *in vitro*. The antigen-specific induction of Tregs and their suppressive capacity against self-reactive T cells is crucial in inducing peripheral tolerance. (Groux *et al.*, 1997; Roncarolo *et al.*, 2001; Maloy and Powrie, 2001; Levings *et al.*, 2006; Kim *et al.*, 2007) Here, immunosuppressive drugs such as rapamycin targeted key functions of DCs, and thus may help towards developing new therapeutic strategies to target immunological diseases such as autoimmunity and allergic diseases. (Hackstein and Thomson, 2004) Foxp3 is the master regulator of immunological tolerance in Tregs and plays a critical role in the maintenance of immune homeostasis. (Brunkow ME *et al.*, 2001; Rudensky, 2011) Foxp3 K.O. mice develop severe autoimmunity with multi-organ inflammation. (Brunkow ME *et al.*, 2001) Our studies focused on $CD4^+$ $CD25^{hi}$ $Foxp3^+$ cells that have been reported as the most abundant and critical Treg subset in maintaining tolerance, though also $CD4^+$ $CD25^{lo/-}$ $Foxp3^+$ cells have been described to suppress $CD4^+$ T cell proliferation. (Fontenot *et al.*, 2005)

Rapamycin

Prior studies suggested that the mTOR inhibitor rapamycin promotes the capacity of DCs to induce and enhance tolerance rather than effector responses. Dendritic cells with these characteristics are denoted tolerogenic and are considered to be in a more immature developmental stage. (Maldonado and Andrian, 2010) This immature stage may help explain their function to expand Tregs, and also impair the expansion of Teff cells. (Turnquist *et al.*, 2007) Interestingly, rapamycin selectively favors the expansion of the $CD4^+$ $CD25^+$ $Foxp3^+$ population *in vitro* and *in vivo*, but also stimulates DCs to increase the expansion of $CD4^+$ $CD25^+$ $Foxp3^+$cells. (Hackstein and Thomson, 2004; Battaglia *et al.*, 2005 and 2006; Turnquist *et al.*, 2007; Keever-Taylor *et al.*, 2007; Kang *et al.*, 2008) It is still not entirely clear how rapamycin specifically acts on T cells, but both mTOR kinase complexes, which are inhibited by rapamycin, are capable of inhibiting $Foxp3^+$ expression in $CD4^+$ T cells, whereas Th1, Th2, and Th17 cells get either regulated by mTORC1 or mTORC2. This is suggestive that mTORC1 and mTORC2 regulate $CD4^+$ $CD25^+$ $Foxp3^+$ generation. Inhibition of both complexes results in increased TGFb sensitivity in culture

and naïve T cell-Treg differentiation also occurs with lower levels of TGFb. (Delgoffe *et al.*, 2009; Gabryšová *et al.*, 2011; Powell *et al.*, 2012)

To rule out the possibility of direct action of rapamycin on T cells in cocultures, a time-consuming protocol was chosen that diluted rapamycin to at least 1:400.000 in all experiments from our treatment concentration in five separate washing steps before adding them to cultures with naïve OT-II Foxp3-GFP T cells. Though, we cannot entirely exclude direct interaction of rapamycin on T cells, but owing to the high dilution it may only had a minor effect on T cells. The effect of the argument, if it were accepted that T cells directly got affected by rapamycin, would not explain the vast differences in Treg induction between different DC subsets discussed later. Therefore, we are certain that even if a minor effect of rapamycin residues is given on T cells, it is not sufficient to affect the outcome.

Control groups

In our studies, we choose to study four groups of antigen-pulsed DCs (naïve control DCs, LPS-treated DCs, rapamycin and TGFb-treated DCs, and one group where TGFb and IL-2 was added to naïve control DCs). The naïve control DC and LPS treated DC group displayed low intrinsic induction capacity. It is known that TGFb not only drives Foxp3 expression, but is necessary for $CD4^+$ $Foxp3^+$ Treg induction. (Gabryšová *et al.*, 2011; Kretschmer *et al.*, 2005; Chen *et al.*, 2003) Likewise, IL-2 is a crucial cytokine for Treg expansion and can be physiologically provided by T cells once they are stimulated by DCs. (Yamazaki *et al.*, 2007) Therefore, we observed maximum Treg induction by addition of these cytokines in excess at day 0 as expected. Unlike stimulation with LPS which causes immature tissue-resident DCs, such as splenic DCs, to mature immunologically. Resulting in poor antigen uptake and migratory activity. (Granucci *et al.*, 1999; Maldonado and Andrian, 2010) Consequently, the stimulation with LPS causes diminished Treg induction compared to naïve control DCs and was therefore used as a negative control in our experiments. Our results fit well with literature and LPS-treated DCs activated less $Foxp3^+$ Tregs on average compared to naïve DCs.

Comparability of experiments

To maintain the same conditions in all experiments, we matched genders and age of DC donor and T cell donor mice, applied the same DC/T cell ratios for *in vitro* cultures, stained OT-II Foxp3-GFP mice to ensure proper TCR specificity of naïve T cells, and sacrificed Flt3L mice for DC isolation at day 14. All other protocols were also not altered. Still, we observed great variation between experiments, though these differences always affected

all four groups. In brief, when the control DC group showed low Treg induction, the it-DC group proportionally also stimulated less Tregs, but still was always higher than the control group (Fig. 20D). Despite these differences in Treg % induction, the outcome when we directly compared x-fold-changes of Foxp3$^+$ Tregs of it-DCs compared to the control DC group was very consistent (Fig. 20C).

4.1.1 Dendritic cell subsets

Though there is consensus that rapamycin affects DCs and T cells, the role of the different tolerogenic DC subsets and their capability to promote Tregs is less well understood. (Maldonado and Andrian, 2010). Until now, none of the spleen DC subsets could be assigned a specific role in their capacity to induce regulatory T cells.

As we were able to show successfully that CD11c$^+$ it-DCs from spleen promote the development of Foxp3$^+$ Tregs, we further investigated our total CD11c$^+$ population and sorted four CD11c$^+$ subpopulations by flow cytometry (CD4$^+$, CD8$^+$, CD11b$^+$, pDC, and CD4$^-$ CD8a$^-$ CD11b$^-$ (triple negative (TN)) to assess their individual potential in antigen-specific T cell stimulation. The same protocols and procedures applied for CD11c$^+$ DCs were maintained for subsets including four groups for every subset tested.

In all subsets it-DCs showed superior Treg induction compared to control and LPS-DCs, although the intrinsic activity to stimulate T cells differed significantly between subsets. Though pDCs (it-DCs) also showed higher Foxp3$^+$ Treg induction, pDCs displayed very low total cell numbers, poor growth and viability in cultures. (Fig. 21). These findings confirmed previous results with CD11c$^+$ DCs and all subsets, except pDCs, gained to some extent an advantage in Treg stimulation through rapamycin and TGFb stimulation.

Triple negative and CD8a$^+$ DCs

The CD8a$^+$ and CD4$^-$ CD8a$^-$ CD11b$^-$ (TN) DC subsets showed significant increases in percentages of Foxp3$^+$Tregs. TN DCs are the most abundant subsets (~52%) isolated from Flt3L spleens, followed by CD11b$^+$ (25%) and CD8a$^+$ DCs (8%; Fig. 16). In non-Flt3L mice, CD8a represented 29% of DCs and TN 1%. CD8a$^+$ DCs also showed the highest intrinsic activity (i.e. control DCs) in expanding Tregs of all subsets. This intrinsic activity was more than two-fold increased compared to TN as the second ranked DC. (Fig. 23B)

To understand why CD8a$^+$ it-DCs excel in Treg induction, we need to know that the hallmark functions of CD8a$^+$ DCs are IL-12 and INF-a secretion as well as cross-presentation. IL-12 induces INF-γ production that further favors Th1 differentiation. (Hochrein *et al.*, 2001; Trinchieri, 2003) CD8a$^+$ DCs IL-12 production can be stimulated by various pathways such as MyD88. Rapamycin inhibits MyD88 and mice deficient in the

MyD88 pathway show prolonged survival of skin grafts what indicates development of immunological tolerance. (McKay *et al.*, 2006; Chang *et al.*, 2013) Moreover, Fltl3 DC expansion is mTOR dependent and rapamycin impairs Flt3L induced DC maturation, whereby $CD8a^+$ DCs are among the most affected DCs. (Sathaliyawala *et al.*, 2010) DCs persistently express Flt3 during their development and it is maintained on terminally differentiated DCs as well. (Liu *et al.*, 2009; Schmid *et al.*, 2010) Recently, it was shown that proliferating $CD4^+$ OT-II cells are capable of producing Flt3L to a greater extent than $CD8^+$ T cells, B cells, or NK cells and directly contribute to DC development. (Saito *et al.*, 2013) It is unclear to what extend the inhibition of Flt3 affects function and *ex vivo* maturation of $CD8a^+$ DCs, in particular if we consider them as not terminally differentiated. In summary, we speculate that rapamycin stimulation locks $CD8a^+$ DCs in a developmental stage that allows increased Treg expansion while further Flt3 maturation or development is inhibited.

Lung DCs and CD8a

Results from an independent experiment where macrophages and DCs from lung tissue and BAL fluid were isolated suggested that tissue resident lung-DCs, contrary to macrophages, have an increased ability to activate $Foxp3^+$ Tregs compared to naïve splenic $CD11c^+$ DCs. In this experiment, we did not distinguish between the two major lung DC populations $CD103^-$ $CD11b^{hi}$ and $CD103^+$ $CD11b^{lo}$. (Kopf *et al.*, 2014) Nonetheless, $CD103^+$ DCs are considered migratory DCs that carry antigen from peripheral tissues to secondary lymphoid organs. Interestingly, it is assumed that the $CD103^+$ DC subsets in peripheral tissues are equivalent to $CD8a^+$ DCs in lymphoid tissues. (Satpathy *et al.*, 2012) This observation reinforces a functional relationship between $CD8a^+$ and $CD103^+$ lung-DCs and indicates a common superior capability to stimulate antigen-specific Tregs.

TN and CD8a lineage

The triple negative subset has been rarely studied in the past. However, to further define and assign closer to its ancestor, recent literature was studied extensively (see pre-DC in Introduction) and may give a clue about a closer ancestor of this subset and its relation to $CD8a^+$ DCs. (Naik *et al.*, 2006)

Here, we suggest that TN DCs are a lineage that separate early in their development mostly due to the reported surface markers $CD24^{lo}$ $CD205^-$ on these precursors, whereas $CD8a^+$ DCs were mostly generated from $CD24^{hi}$ $CD205^+$ cells. Moreover, it was shown that CD11b is positive early in the development of DCs.

Tumor-bearing (Flt3L) mice show a major increase in total cell numbers of CD8a$^+$ DCs and TN DCs, despite the fast turnover rate of about 36h of CD8a$^+$ compared to CD4$^+$ and CD11b$^+$ DCs. Suggesting that TN DCs could interchange to CD8a$^+$ DCs. Furthermore, in our experiments, we received a significant increase in Treg expansion of TN DCs and CD8a$^+$ DCs compared to all other investigated subsets, which is indicative of a functional common feature.

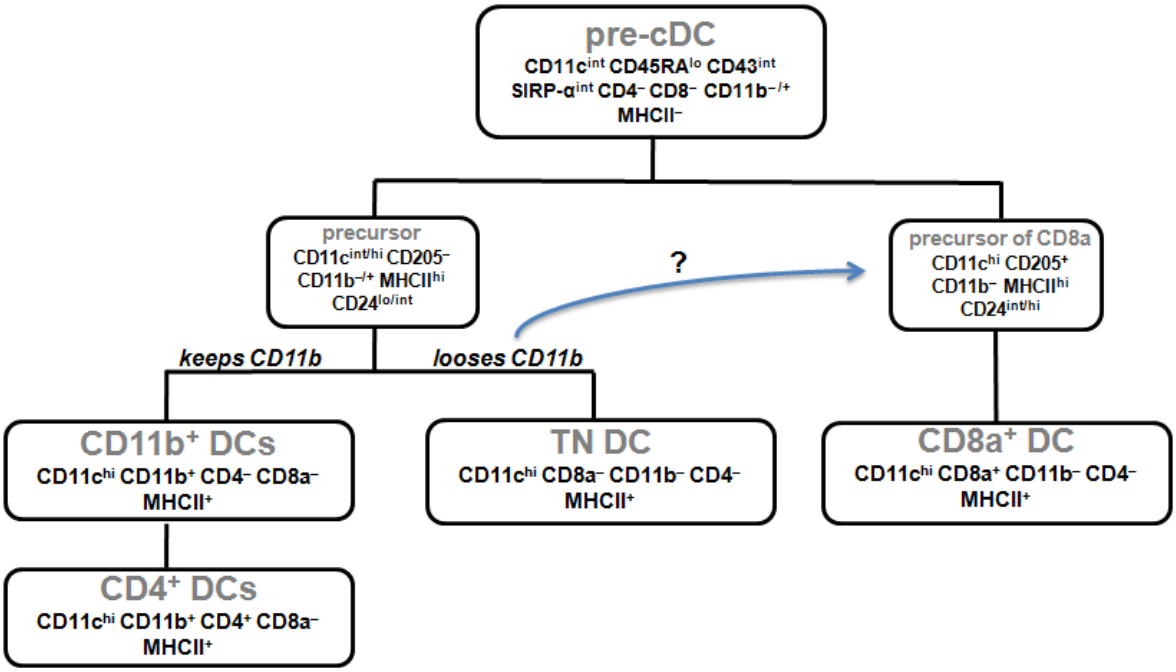

Fig. 42 Proposed DC subsets relationships of four of our investigated murine subsets (CD11b$^+$, CD4$^+$, TN, and CD8a$^+$ DCs). The blue arrow indicates that it is unclear if TN DCs are able to interchange to CD8a$^+$ DCs.

However, we consider the TN as a DC progenitor that might be able to interchange or develop to CD8a$^+$ DCs. This can be argued by the fact that significant morphological differences in long-time video imaging with naïve T cells were observed in CD8a$^+$ DCs that were entirely obsolete in TN DCs despite both DCs revealed an increased capacity to induce Foxp3$^+$Tregs *in vitro*. Likewise, it needs to be mentioned that rapamycin may lock TN DCs in its current maturation state and does not allow further phenotypical and/or functional development. Additionally, gene expression analysis revealed that CD8$^-$ CD4$^-$ and CD4$^+$ CD8$^-$ more resemble each other than CD8a$^+$ DCs in terms of gene expression patterns, whereby CD8$^-$ CD4$^-$ consist of at least two more subpopulations. Therefore, CD8a$^+$ DCs are considered as a more distant lineage. (Edwards *et al.*, 2003) CD205 is a DC maturation marker and higher expressed on matured DCs. (Butler *et al.*, 2007) Thus, we consider TNs less mature than CD8a$^+$ DCs. Nonetheless, we cannot exclude that TN precursors or TN DCs itself are able to interchange to CD24^{hi} CD205$^+$ (Fig. 42, blue arrow), which would serve as direct precursor of CD8a$^+$ DCs.

4.1.2 lung macrophages

According to a recent study, lung macrophages contributed to immunolgical tolerance in lung tissue by increased activation of Tregs. (Soroosh *et al.*, 2013) Our results were not able to confirm these findings in lung tissue resident macrophages or macrophages in BAL fluid, but revealed that lung-DCs have a higher intrinsic activation of Tregs compared to non rapamycin treated control DCs. Here, we also did not distinguish between the two major lung cDC populations $CD103^{-}$ $CD11b^{hi}$ and $CD103^{+}$ $CD11b^{lo}$ (Kopf *et al.*, 2014), but distinction into subsets is certainly of interest in further studies. Whether this finding is directly translatable to human macrophages, awaits further experimentation, as it is difficult to receive healthy human lung tissue for follow-up studies in humans.

4.1.3 *in vivo* it-DC mediated $Foxp3^{+}$ Treg induction

We showed that adoptively transferred naïve (i.v.) OT-II cells get activated and convert to antigen-specific $Foxp3^{+}$ $CD25^{hi}$ Tregs *in vivo* by adoptively transferred (i.v., f.p., and i.n.) ovalbumin pulsed $CD11c^{+}$ DCs in lymph nodes and spleen in transgenic 2D2 mice. The TCR of 2D2 mice is specific and limited to recognize the myelin oligodendrocyte glycoprotein (MOG) and CD45.2 T cells from 2D2 mice are unable to recognize ovalbumin. (Bettelli *et al.*, 2003). Whereas OT-II Foxp3-GFP T cells only recognize processed ovalbumin peptides presented by DCs and are the sole T cell population that is expected to be primed by our donor DCs. $CD11c^{+}$ stimulated DCs (it-DCs), LPS DCs, and a naïve control DCs were compared. Maximal cell numbers of $Foxp3^{+}$ $CD25^{hi}$ expressing $CD4^{+}$ CD45.1.2 T cells were observed with it-DCs in all tissues, except blood where no induction was detected in any group. Though in lymph nodes and spleen, total Treg cell numbers were higher compared to control, only lymph nodes were able to show a specific $Foxp3^{+}$ $CD25^{hi}$ Treg induction by not expanding $Foxp3^{-}$ $CD25^{+}$ cells simultaneously.
During our studies, we did not find a publication that was able to verify that antigen-specific induction of $Foxp3^{+}$ T cells by DCs takes place *in vivo*. Hence, to evaluate our findings in a more clinical setting, the next step was to determine if it-DCs were capable of inhibiting allergic inflammation in a mouse model of asthma.

4.1.4 it-DC therapy in OVA-based asthma models

Here, we used a mouse model of asthma, by examining the acute allergic response to an inhaled allergen (ovalbumin). (Nials and Uddin, 2008) We induced an acute asthma-like reaction by systemic sensitization (ovalbumin+$AlOH_3$) and challenged mice with aerosolized ovalbumin. Mice that are exposed to aerosolized antigen such ovalbumin (in the absence of adjuvants such as $AlOH_3$) usually develop airway tolerance mediated, in part, by $Foxp3^{+}$ $CD4^{+}$ T cells. (Ostroukhova *et al.*, 2004) The aim of this experiment was to

identify if intranasal administration of a particular subset of it-DCs ($CD8a^+$ DCs, TN DCs, $CD11c^+$ panDCs) was capable of inhibiting allergic airway inflammation by creating immunological tolerance through the induction of $Foxp3^+$ Tregs.

By adoptively transferring it-DCs in challenged mice (i.n.) we showed that $CD8a^+$ it-DCs were able to inhibit an allergic response by lowering eosinophils count in BAL fluid and lung tissue (Fig. 30). By contrast, no meaningful differences between the control group (no DCs), $CD11c^+$ panDCs, and TN DCs were detected. An *in vitro* quality control confirmed the superior potential of the transferred $CD8a^+$ it-DCs that were above average than previous *in vitro* experiments and therefore demonstrated the highest Treg conversion (46.1%), followed by TN it-DCs (24.4%; below average). We cannot exclude that this might be also a reason why TN DCs were unsuccessful in inhibiting airway inflammation in this experiment. Though the TN subset generated high Treg induction in *in vitro* experiments, we can only speculate why these data do not match with this *in vivo* experiment. We know that $CD8a^+$ DCs are considered as non-migratory resident DCs but also have a functional relationship to migratory $CD103^+$ DCs that reside in lung tissue. Interestingly, it has been proposed that the tolerogenic potential is highest for pDCs followed by $CD103^+$ DCs and $CD8a^+$ DCs. (Shortman and Heath, 2010; Maldonado and Andrian, 2010) Our results suggest that the administration of it-DCs as a potential therapeutic strategy to battle allergic diseases is a promising approach that needs to be further investigated to confirm our most recent findings.

4.2 Human experiments

In our human studies, we focused on four blood-borne circulating DC subsets ($CD141^+$, $CD16^+$, $CD1c^+$, pDC). Additionally, blood monocytes were isolated and converted to monocyte-derived DCs (moDCs). All DCs were cocultured with autologous anti-CD3-stimulated polyclonal CD4 T cells from the same donor. Due to the difficulty in resolving true $Foxp3^+$ Tregs from $Foxp3^+$ expressing activated conventional CD4 T cells, additional markers including CD45RO (maturation marker), CD45RA, and CD25 (as well as Foxp3) were used to identify activated T cells and Tregs. (Borsellino *et al.*, 2007) In preliminary experiments that focused on establishing culturing protocols with human source materials, we noted a generalized decline in viability and total cell yield in DC-T cocultures when cultured in the absence of human serum. In the presence of human serum, the expansion and viability of cells in culture dramatically increased in nearly all DC-T culture conditions. This allowed us for the first time to identify discrete populations of $Foxp3^+$ $CD25^{hi}$ Tregs in moDCs and $CD16^+$ DCs. The cytokines or growth factors present in human serum that may have been responsible for the increase are not completely known.

4.2.1 $CD141^+$ and $CD1c^+$ DC

$CD141^+$ DCs are considered as functionally related to $CD8a^+$ DCs due to their proposed function in self-tolerance and increased production of IL-12. (Crozat *et al.*, 2010; Kushwah and Hu, 2011) Due to low abundance and difficulty in isolating, they have rarely been studied in detail. Here, we were able to directly compare $CD141^+$ with the other three common blood DC subsets. We observed that CD141 it-DCs seem to be unable to activate naïve $CD4^+$ T cells but rather locks all T cells in a more naïve state ($CD45RO^-$ $CD25^-$). Recently, it was suggested that $CD141^+$ DCs rather play a role in antiviral immunity and may be inferior in IL-12 secretion compared to CD1c. (Hémont *et al.*, 2013; Nizzoli *et al.*, 2013)

$CD1c^+$ DCs have been reported to be the functional equivalent of murine $CD11b^+$ DCs (Crozat *et al.*, 2010; Robbins *et al.*, 2008) and recently suggested to be the major IL-12 secreting blood DC for naïve T cells.(Nizzoli *et al.*, 2013) However, no $CD45RO^+$ $CD25^+$T cell activation was observed by $CD1c^+$ it-DCs and the naïve $CD1c^+$ control showed superior induction over it-DCs. Remarkably, the positive control group was capable of inducing $CD4^+$ $CD45RO^+$ $CD25^{hi}$ $Foxp3^{hi}$ cells.

Our data do not argue against the participation of $CD141^+$ and $CD1c^+$ DCs in tolerance, but rapamycin may block pathways in these DC subsets that abrogates or diminishes their tolerogenic nature. $CD141^+$ it-DCs are incapable of activating naïve cells where the entire

naïve T cell population was not expressing CD25 or CD45RO. Likewise, $CD141^+$ it-DCs also did not show morphological changes in live cell imaging and underwent apoptosis within 48h, whereby $CD1c^+$ it-DCs were viable for over 96h in culture. Although $CD1c^+$ it-DCs were viable in live cell imaging over a prolonged time and stimulated CD25 and CD45RO expression, but $CD1c^+$ control DCs still activated naïve T cells to a greater extent than it-DCs.

4.2.2 pDCs

Though it is suggested that pDCs have some role in immunological tolerance control- and rapamycin and TGFb- treated pDCs did not activate T cells, where over 99% of all $CD4^+$ T cells were still $CD45RO^-$ $CD25^-$. Similar to murine pDCs, treatment of human pDCs with rapamycin and TGFb did not show an effect on T cell proliferation or activation. *In vivo* live-cell imaging, affirmed that the huge majority of pDCs seem to be dead within about 24h in cultures, which is in sharp contrast to the other researched DC subsets. In view of poor growth and viability, cultivation conditions for pDCs may have contributed to the low activation of T cells and their conversion into Tregs. Still, pDCs are known as the major DC subset in viral responses rather than immunological tolerance. (Adam *et al.*, 2005)

4.2.3 $CD16^+$ DCs and moDCs

Here, we isolated $CD16^+$ $CD14^-$ $CD11c^{hi}$ Lin^- blood DCs that have been described to have characteristics of monocytes and often denoted as pro-inflammatory monocytes, though classical monocytes are $CD14^+$. $CD16^+$ DCs that express high levels of TNF-α , IL-6, and IL-10. $CD16^+$ DCs are similar to murine monocytes due to their high expression of SIRP-α and CD11b, but not CLEC9A. (MacDonald *et al.*, 2002; Belge *et al.*, 2002; Mittag *et al.*, 2011) MacDonald et al. showed that moDCs and $CD16^+$ DCs share features such as low to none cutaneous lymphocyte antigen (CLA) expression, whereas it is widely expressed by other DCs. Moreover, it was reported that moDCs express profiles that are intermediate to $CD16^+$ DCs. (MacDonald *et al.*, 2002; Collin *et al.*, 2013) $CD16^+$ DCs excel in cell viability, proliferation, $CD45RO^+$ $CD25^{hi}$ T cell activation, and $Foxp3^+$ $CD25^{hi}$ Treg stimulation over all other human blood DC subsets.

Our experiments revealed that moDCs have a tremendous intrinsic activity to stimulate naïve T cells, but preliminary experiments without human serum in cultures lead to a generalized activation of all naïve T cells that made it difficult to draw further conclusions. This means that all four groups showed the same unspecific, but strong T cell activation. However, the addition of human serum led, in particular for the rapamycin treated moDCs group, to the development of three T cell subpopulations; an activated $CD45RO^+$ $CD25^{hi}$

subset with increased Foxp3 expression, an intermediate subset of CD45RO^{int} CD25^{int} cells with lower Foxp3 expression and a CD45RO^{-} CD25^{-} subset with no Foxp3 expression. These three populations may represent different developmental stages. Still, if these would be solely different developmental stages, we would expect to see a homogenous continuous profile instead of clearly identifiable populations. Additionally, a distinct population of Foxp3^{+} CD25^{hi} was identified in it-DCs. This is suggestive of the following: human serum contains cytokines other than IL-2 and TGFb that are mandatory in exploiting the full potential of moDCs in priming T cells. Moreover, in these presumably more physiological conditions *in vitro*, rapamycin cause moDCs to specifically stimulate some T cell subtypes to a higher extent whereas others are inhibited.

In a porcine model, it was shown that blood monocyte-derived DCs (moDCs) induced stronger proliferation in naïve T cells than DCs isolated from blood. (Facci *et al.*, 2010) Furthermore, a murine model revealed that under inflammatory conditions moDCs are a major producer of IL-12 relative to CD8a^{+} cDCs *in vivo*. moDCs IL-12 production is largely dependent on MyD88 signaling which is blocked by rapamycin, as it is in CD8a^{+} murine it-DCs. (Zhan *et al.*, 2010) Taking our results into consideration of CD8a DCs in mouse and human moDCs, we speculate that the inhibition of MyD88, particularly in strong IL-12 secreting DCs, lead to a pronounced Treg activation accompanied by a decreased Th1 response.

Our results showed that CD16^{+} DCs and moDCs are functionally related in their potential to activate T cells as those were the only two human DC subsets that were able to elicit a meaningful T cell activation and detectable Foxp3^{+} CD25^{hi} Treg response. A final classification of the CD16^{+} blood subset is not feasible and further studies need to follow up to characterize marker expression of both DC types. Here, we speculate that moDCs could give rise to the CD16^{+} DC subset in blood. Our results support previous findings that place CD16^{+} and moDCs / monocytes in a closer relationship to each other than to other blood DCs. Interestingly, the small population of CD14^{bright} CD16^{+} monocytes have been identified to be increased in rheumatoid arthritis while CD14^{dim} CD16^{+} were not increased in blood. (Rossol *et al.*, 2012)

In summary, we showed that short-term rapamycin and TGFb conditioning of all murine spleen DC subsets and CD16^{+} as well as moDCs in human strongly favor the differentiation of naïve T cells into the Fopx3 regulatory T cell phenotype *in vitro*, and *in vivo* in mice. Though it has been reported that human blood DCs are similar to its spleen counterparts, our findings indicate that splenic mouse and human blood DCs are not fully functionally equivalent and interspecies differences exist. Although several strategies to

modify T cell activity with modified DCs have been explored, we know little about whether and how DCs induce Tregs, and if they could prevent, treat or cure allergic disorder. The ultimate goal in the treatment of autoimmunity is to reestablish tolerance by potentiating regulatory T cell numbers and/or functions. Comparison of the different subsets in mouse, revealed that it-DCs subsets ($CD8a^+$ and TN DCs) were by far the most effective in antigen-specific Treg induction from which one subset ($CD8a^+$) was able to ameliorate the Th2 response in a classic murine model of allergic asthma by lowering total eosinophil counts.

The identification of the human blood DC subset $CD16^+$ and moDCs, both with similar potentials to stimulate T cells, is a significant advance bringing it-DC mediated therapy into clinical practice. Isolation of patient DCs from blood, pulsing with the disease causing antigen, and reprogramming with rapamycin and TGFb is a promising therapeutic strategy with relatively low regulatory barriers, considering that autologous cells have a low risk of autoimmune mediated rejection and rapamycin is an immunosupressant clinically used in long-treatment for graft rejections. In the light of these novel findings, we hypothesize that it-DCs are useful to treat or prevent other T cell-mediated allergic disorders, such as food allergy. Prior to our studies, outstanding questions of differences in human and mice and subset DC cell activity limited clinical application. This preclinical work in mouse and human provides a rationale for clinical translation of induced tolerogenic DC as a platform for cellular therapy of allergic diseases.

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6 Attachments

Video imaging screenshots are representative of the videos that are digitally available online. Pictures are shown at three or five time points (0h, 24h, 48h, 72h, 96h). However, the attached videos are in 40x and 80x magnification, but screenshots also show videos that were taken for some DC subsets with 20x magnification.

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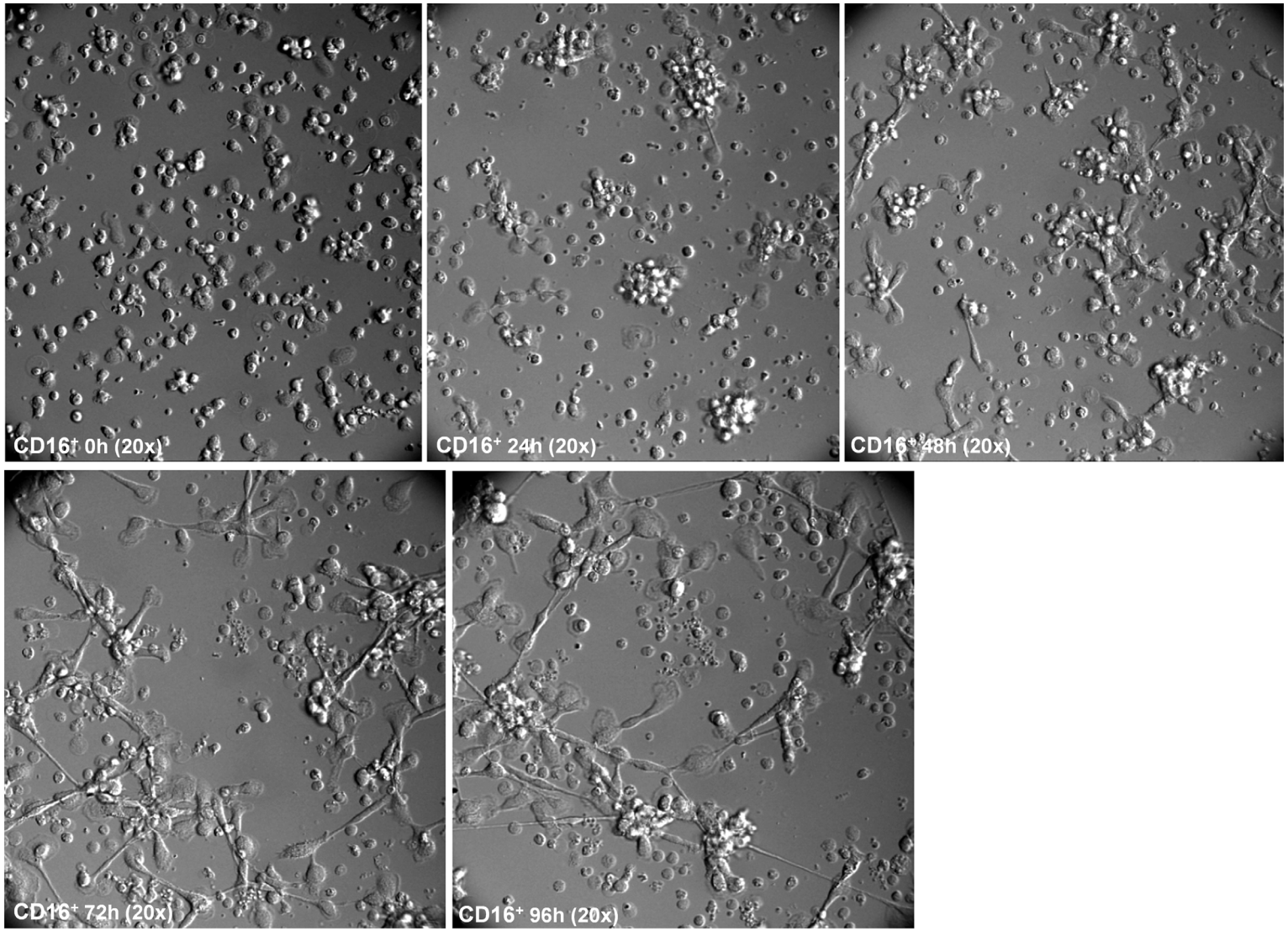
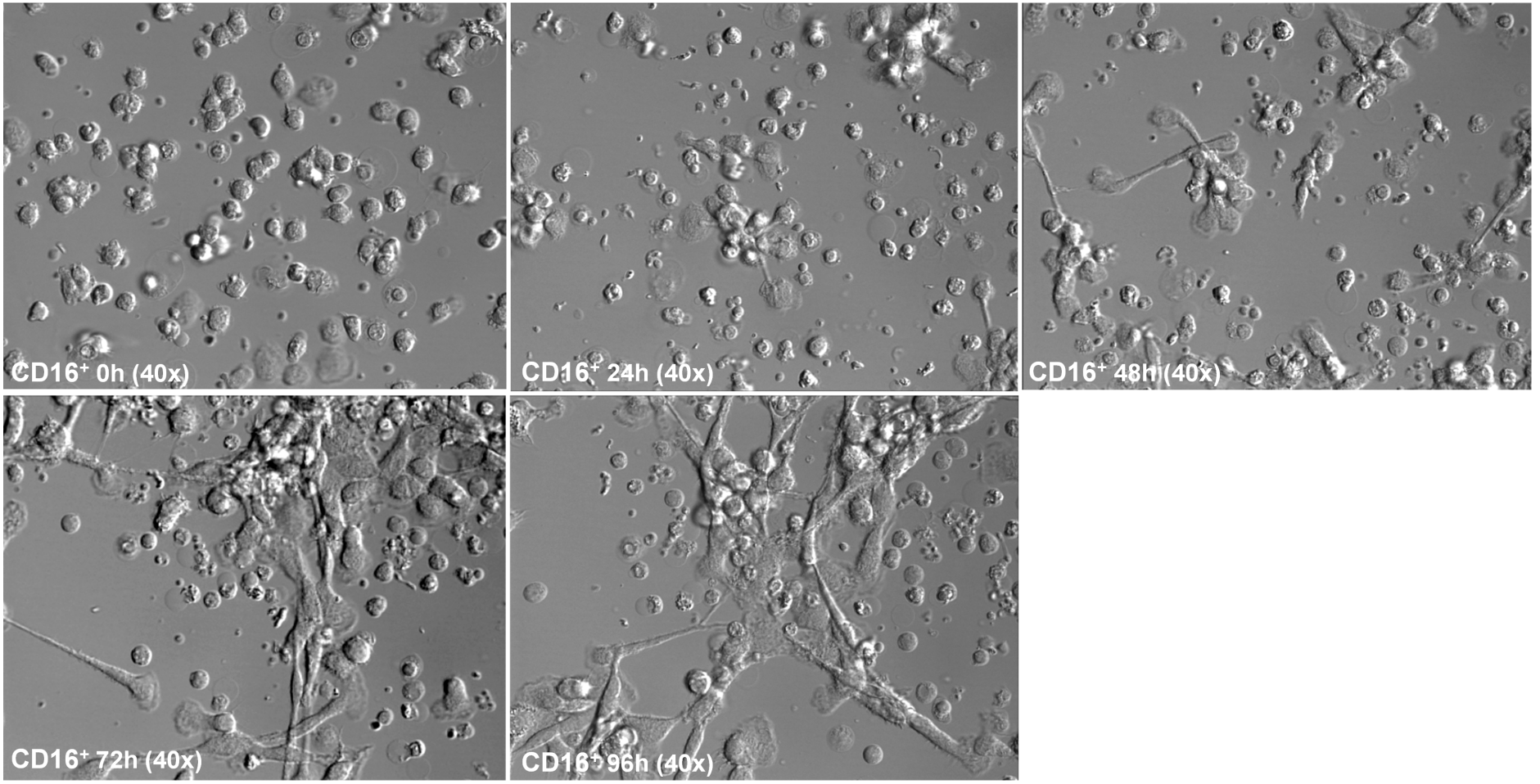
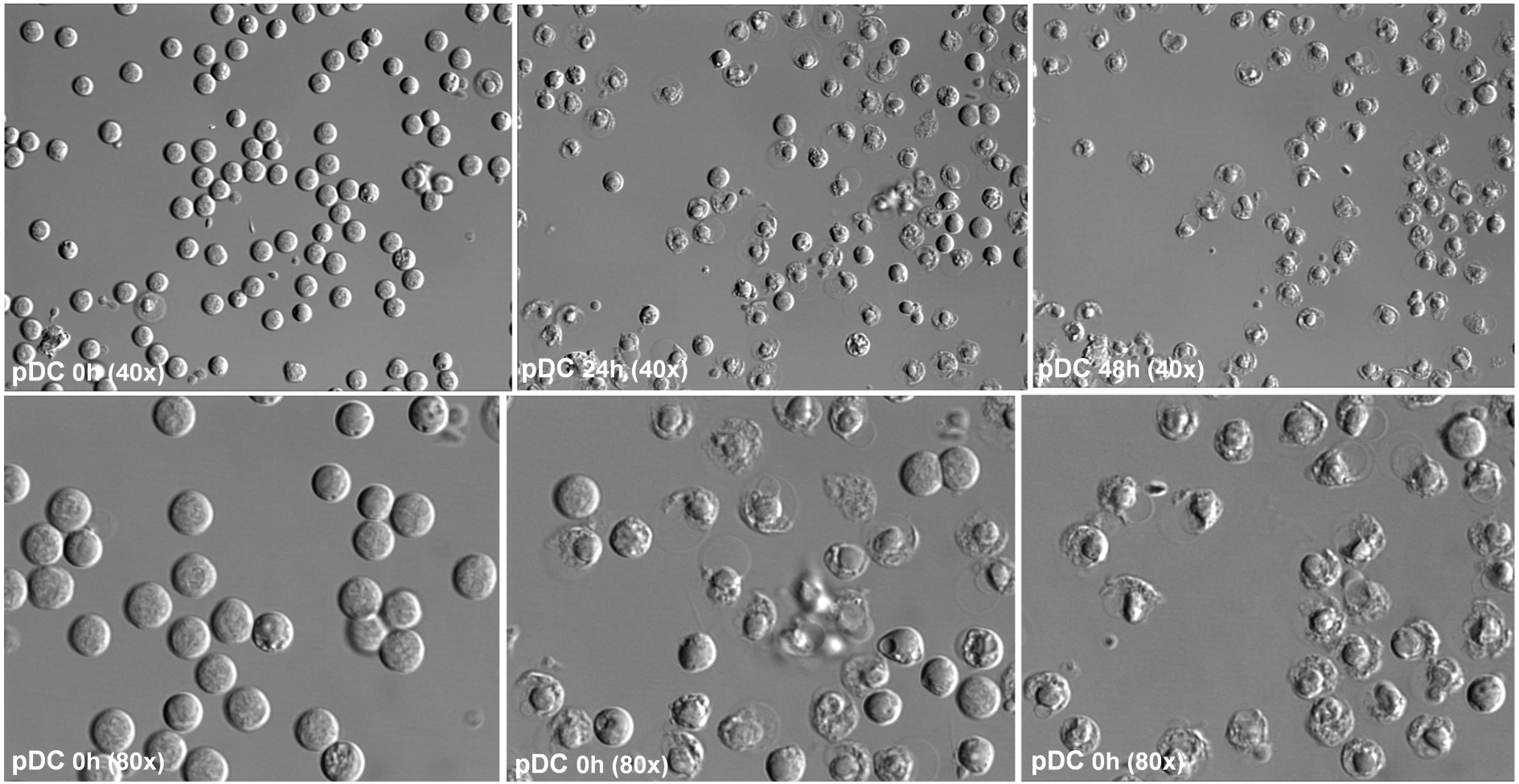
Human CD16$^+$ DC monoculture (20x magnification)



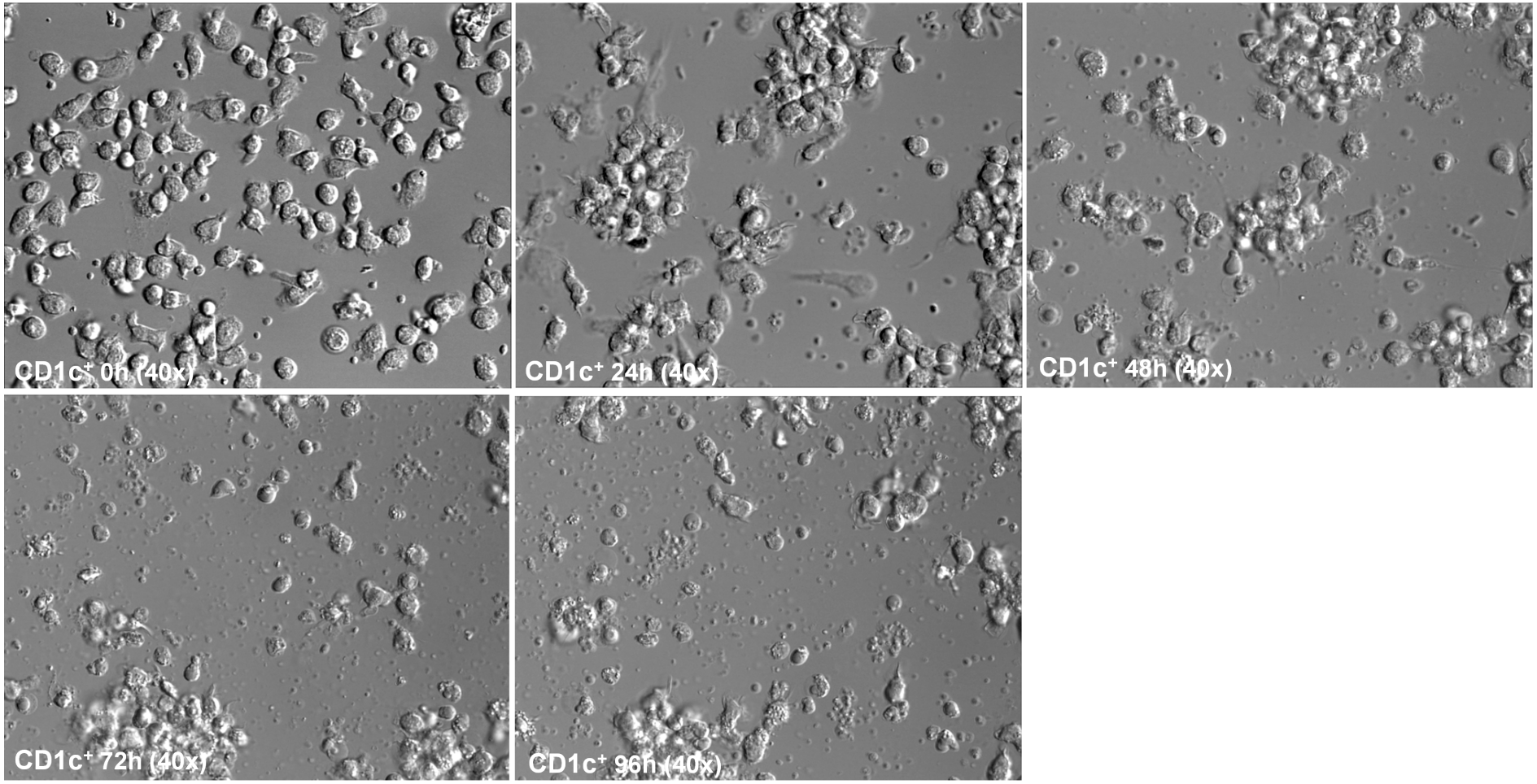
Human CD16^{+} DC monoculture (40x magnification)



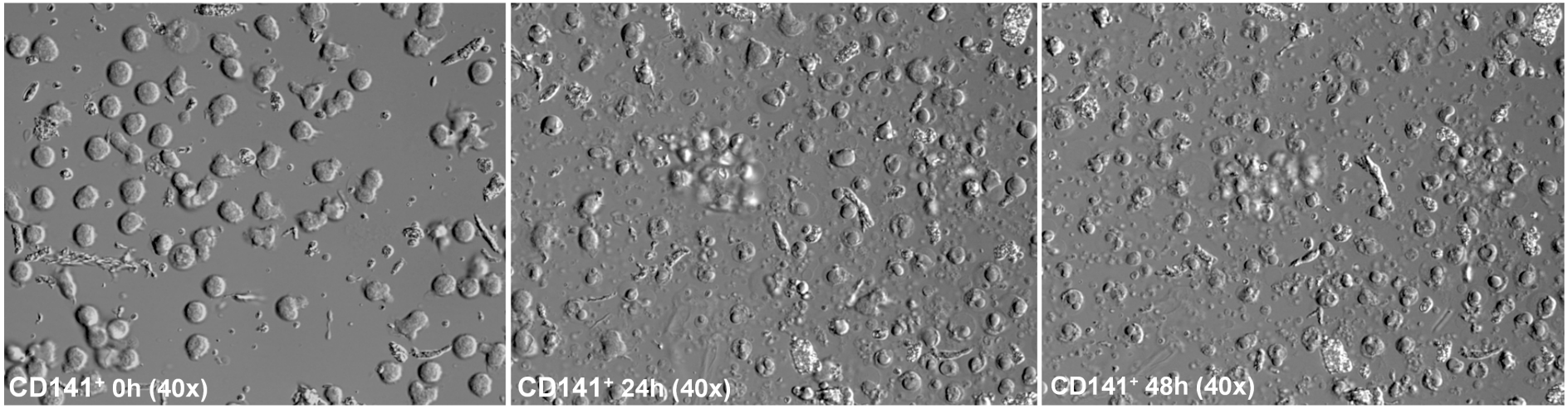
Human pDC monoculture (40x and 80x magnification)



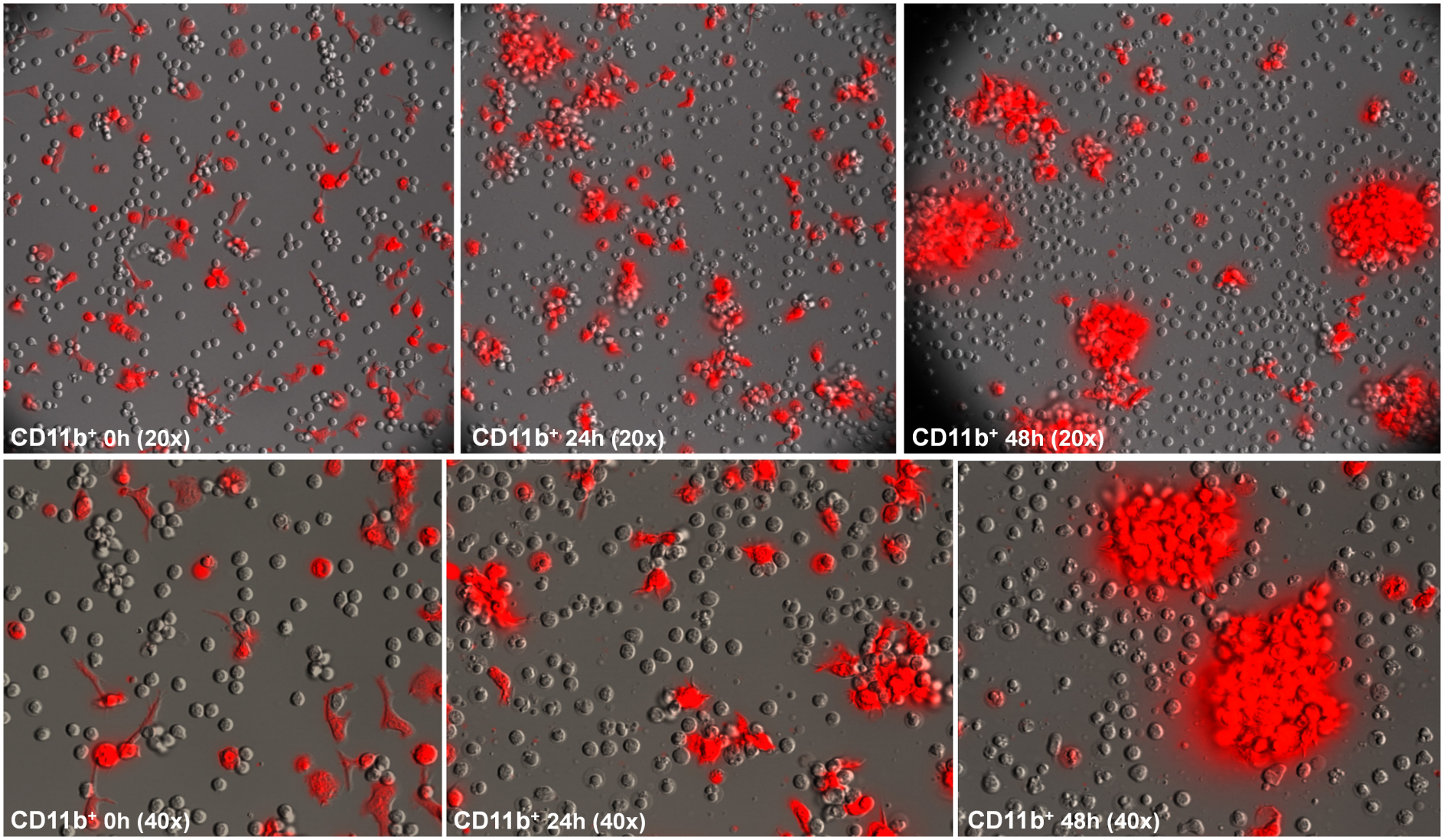
Human CD1c$^+$ monoculture (40x magnification)



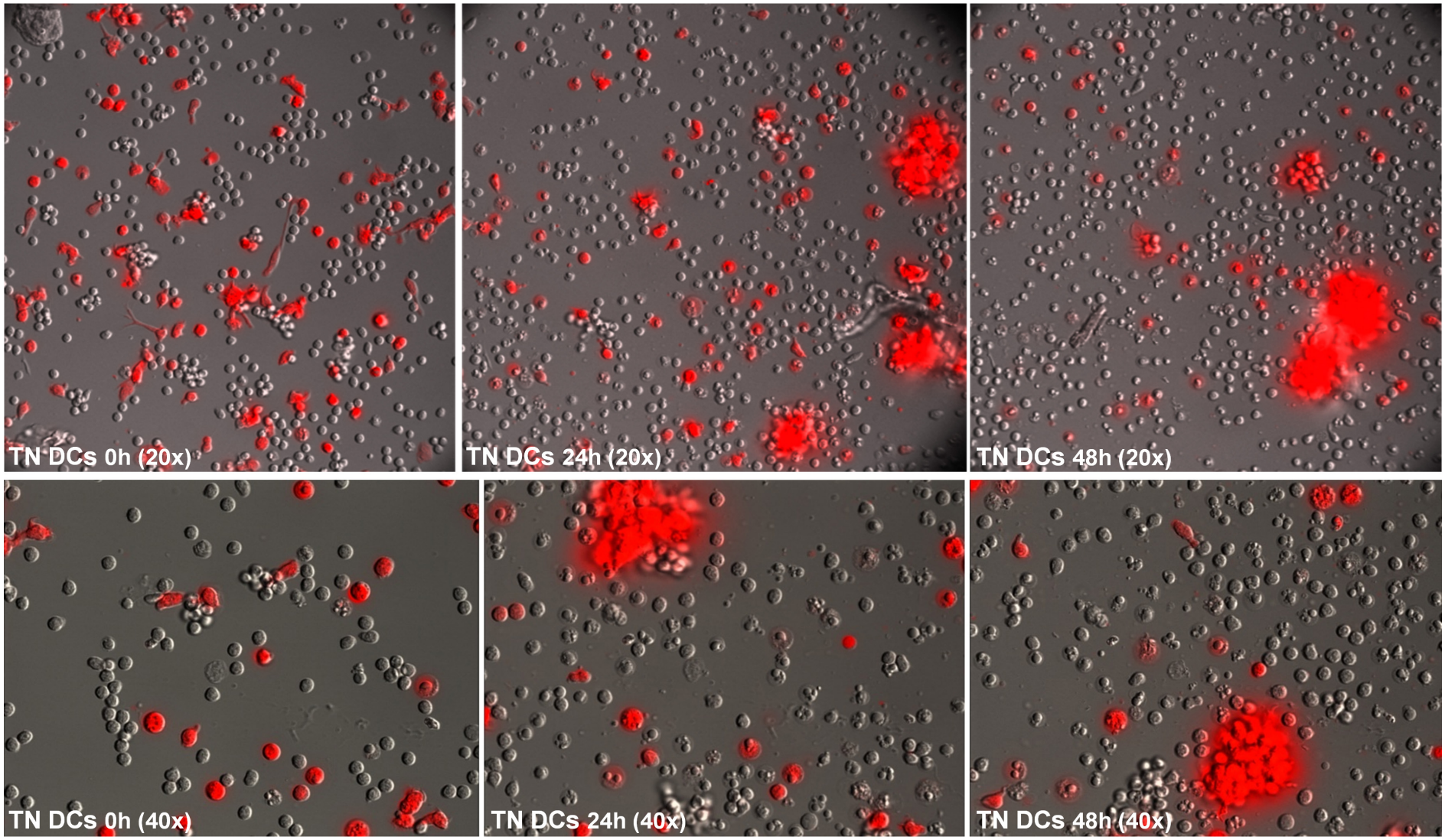
Human CD141^{+} DC monoculture (40x magnification)



Murine CD11b$^+$ DC / T cell coculture. DCs are fluorescing red and none fluorescing cells are CD4$^+$ T cells.



Murine triple negative DC / T cell coculture. DCs are fluorescing red and none fluorescing cells are $CD4^+$ T cells.



Murine CD8a$^+$ DC / T cell coculture. DCs are fluorescing red and none fluorescing cells are CD4$^+$ T cells.