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Dissertação especialmente elaborada para obtenção do grau de Mestre em Investigação Biomédica

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"So long as you write what you wish to write, that is all that matters; and whether it matters for ages or only for hours, nobody can say."

Virginia Woolf, A Room of One's Own, 1929

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Abstract

The naïve CD4 T cell compartment is essential to maintain the immunocompetence of the individual throughout their lifetime. This compartment, generated in the thymus, is sustained, in the periphery, by pro-survival signals and low proliferation, induced by the response to low-level TCR signalling and IL-7 stimulation. Other pathways, namely the Wnt/TCF1 axis, are suggested to be involved in the preservation of the naïve phenotype. However, the naïve CD4 T cells are a heterogeneous population and the impact of the existent heterogeneity on the response to homeostatic signals is yet to be completely understood. Within the naïve CD4 T cell compartment is well-established the presence of CD31-defined subsets, which have different homeostatic proliferative profiles.

Here, we proposed to better understand the role of the CD31 expression in the response to signalling pathways involved in the homeostasis of the naïve CD4 T cell compartment, especially IL-7 and Wnt signalling. Additionally, since Wnt/TCF1 axis has been rarely studied in the context of naïve CD4 T cell homeostasis, we also aimed to investigate the impact of its modulation.

Through our characterization of the CD31-defined subsets, we found the differences in the marker's expression to be subtle, but potentially have implications in the response to stimuli. Moreover, we also identified a new level of naïve CD4 T cell heterogeneity defined by TCF1 expression, although unrelated to CD31. Following with *in vitro* experiments, we found a possible crosstalk between Wnt and IL-7 signalling pathways. Indeed, we found evidence that IL-7 stimulation has an addictive effect on the activation of Wnt signalling, while the Wnt activation hinders the response to IL-7. Moreover, the combined modulation of the Wnt pathway and IL-7 stimulation was shown to induce phenotypical changes, especially in the expression of CD38 and CD95, different between CD31 subsets.

Keywords: Naïve CD4 T cells, T cell homeostasis, CD31, IL-7, Wnt/TCF1 signalling

Resumo

O compartimento humano das células T CD4 naïve é indispensável para manter a imunocompetência do indivíduo durante a sua vida. Esta população de células é fundamental para proteger o sistema contra potenciais futuros antigénios patogénicos, em comparação às células T memória que são criadas para uma resposta mais eficaz a antigénios patogénicos já encontrados pelo sistema. As células T CD4 naïve são geradas no timo, durante o desenvolvimento das células T, e, uma vez maduras, emigram para a periferia, onde circulam entre o sangue e órgãos linfoides secundários. É aqui, nos órgãos linfoides secundários, que as células T naïve são expostas a estímulos homeostáticos que promovem a sua sobrevivência e proliferação. As principais vias de sinalização envolvidas na manutenção periférica deste compartimento são a estimulação tónica do TCR (recetor das células T), promovido pela interação de baixa afinidade com auto-péptidos/MHC (complexo de histocompatibilidade maior), e estimulação com a citocina IL-7. Com o avanço da idade, ocorre uma diminuição do número de células geradas pelo timo e, consequentemente, o sistema imune começa a depender cada vez mais desta manutenção das células T CD4 naïves estabelecida na periferia. No entanto, é importante notar, que a construção do TCR apenas ocorre no timo e, consequentemente, a proliferação periférica, não cria, apenas mantém a diversidade existente do reportório do TCR.

Em contrapartida, o número de células T CD4 naïves também é regulado por outros processos, inclusive, morte celular e diferenciação para o compartimento efetor-memória, aquando do aparecimento de uma infeção. Assim, no contexto de homeostasia deste compartimento, torna-se relevante não só discutir vias de sinalização que promovem a sobrevivência e proliferação, mas também que são responsáveis por manter o fenótipo das células naïve. Dentro deste eixo de investigação, a via de sinalização Wnt/β-catenina/TCF1 tem ganho relevância, devido não só ao seu papel geral na diferenciação celular e manutenção de células estaminais, mas também por estar envolvido em vários processos relacionados com as células T. De facto, a sinalização do Wnt, mas especialmente, a expressão de TCF1, mostra ter um papel importante na manutenção do fenótipo das células T CD4 naïves e, uma vez ativadas, ocorre uma diminuição do seu nível de expressão. Para além disso, esta via de sinalização também tem sido estudada na geração de T_{scms}, células T estaminais de memória, uma subpopulação de células T de memória com propriedades semelhantes a células estaminais. Esta subpopulação de células recentemente identificada é descrita como tendo um fenótipo bastante semelhante às células naïve, com a exceção de também expressar CD95.

O compartimento das células T CD4 naïve é, contudo, bastante heterogéneo e esta heterogeneidade não se reflete apenas em fenótipo, mas também em função e resposta a estímulos homeostáticos. Por exemplo, já dentro do compartimento naïve, existe a distinção entre células convencionais (T_{convs}) e células regulatórias (T_{regs}), duas populações com funções distintas após ativação. Enquanto células naïves, ambas populações respondem de forma semelhante à estimulação com IL-7 *in vitro*, mas, quando analisadas *ex vivo*, as T_{regs}

expressam níveis mais elevados de Bcl-2, pSTAT5 e Ki-67, o que indica que estão continuamente a responder a IL-7. O nosso projeto, contudo, foca-se na heterogeneidade criada pela expressão diferencial de CD31 (ou PECAM-1). De facto, o compartimento de células naïve pode ser divido em duas subpopulações de acordo com a expressão, ou não, de CD31 e que demonstram ter um historial proliferativo diferente. Vários estudos descrevem que as células T CD4 CD31+ passaram por menos ciclos de divisão, em comparação com células $CD31^{neg}$. Além disso, as subpopulações definidas por CD31 demonstram responder de forma diferente a estímulos homeostáticos, inclusive na resposta à estimulação por IL-7 e TCR.

Com este projeto procuramos investigar melhor o papel da expressão de CD31 na resposta das células T CD4 naïve aos estímulos homeostáticos do TCR e IL-7, mas também à sinalização do Wnt. Para tal, propusemo-nos, primeiro, a caracterizar as subpopulações naïves definidas pela expressão CD31, CD31+ e $CD31^{neg}$, em amostras emparelhadas de sangue e amígdalas, um exemplo de órgão linfoide secundário. A quantificação da frequência de células que expressa CD31 em populações T CD4 de memória e naïves, mostrou, de forma esperada, que células naïves têm uma elevada percentagem de células CD31+, enquanto nas células de memória baixa drasticamente. No, entanto, nas T_{scms} encontrámos um padrão de expressão diferente das restantes células de memória, apresentado apenas uma perda parcial de células CD31+. Na análise da expressão de marcadores de células T_{convs} CD4 naïves, observámos que, de forma geral, a subpopulação CD31+ têm uma expressão mais elevada de marcadores identificadores células T naïves. Por outro lado, as células $CD31^{neg}$ revelam ter uma maior frequência de células que expressam marcadores de ativação/*homing*, suportando a ideia de que esta subpopulação recebe mais estimulação pelo TCR. Análise não-supervisionada das amostras revelou a existência de subpopulações definidas por diferentes níveis de expressão de TCF1, sem relação com a expressão de CD31. Observamos, nomeadamente, a existência, presente em sangue e amígdalas, de uma população $TCF1^{neg}$ e, apenas presente em amígdalas, de células $CD69+CD127^{low}TCF^{high}$.

Adicionalmente, realizamos experiências *in vitro* de estimulação da sinalização do TCR e IL-7, em curta duração (3 horas), na presença de diferentes moduladores de CD31. A modulação foi feita em células CD4 T_{convs} totais, sorteadas magneticamente, com o intuito de comparar parâmetros de resposta entre as subpopulações definidas por CD31. Infelizmente, esta experiência de forma geral revelou-se inconclusiva devido a vários fatores experimentais inesperados e, otimizações ao design experimental serão necessárias.

Para além disso, procurámos investigar o impacto da sinalização de Wnt no compartimento de células T CD4 naïve e verificar se as subpopulações CD31+ e $CD31^{neg}$ respondem de forma diferente. Para tal finalidade, executámos culturas de células T CD4 naïves, com curta duração (3 horas), na presença de moduladores de via Wnt e estimulação da sinalização de TCR e IL-7. Encontramos alterações na expressão de marcadores da resposta a IL-7, CD127 e CD25, na presença do inibidor de GSK3, ativador da via Wnt. De forma semelhante, executámos culturas de longa duração (7 dias) na presença de moduladores da via Wnt e estimulação de IL-7.

Após os 7 dias de cultura, análise à resposta a IL-7 revelou que a ativação do Wnt impede a descida da expressão CD127 e o aumento da expressão de Bcl-2, o que sugere uma obstrução na sinalização da IL-7. Em alternativa, é também possível que a inibição de GSK3 interfira diretamente nos mecanismos de autorregulação da expressão de CD127 após sinalização. No entanto, este efeito é acompanhado por aumento na proliferação de células, especialmente células T CD4 naïves CD31+. A modulação da via Wnt também resultou em alterações fenotípicas, especialmente na presença de IL-7, nomeadamente na expressão de CD38 e CD95. De facto, nós observamos que, na presença de IL-7, ativação do Wnt diminui a expressão de CD38, enquanto, a inibição da via aumenta a expressão do marcador. Curiosamente, este efeito é mais evidente em células T CD4 naïves $CD31^{neg}$. Dado que DKK-1 é um inibidor fisiológico da via Wnt, que aumenta sistemicamente com a idade, e o papel do CD38 na disfunção metabólica de células imunes, é possível que esta seja via reforçada com a idade que contribui para o envelhecimento de células CD4 T naïve. O aumento de expressão de CD95 foi observado tanto na presença individual de IL-7 e do ativador da via Wnt, todavia, na presença de ambos, este efeito mostra ser aditivo e intensificado, especialmente nas células naïves CD31+. Apesar desta observação ser interpretada por outros como levando à geração de T_{scms}, tais estudos foram efetuados na presença de estimulação pelo TCR e está ainda por demonstrar que o fenótipo adquirido seja estável e que as células assim geradas de facto representem T_{scm}. Análises adicionais à função efetora e fenótipo será necessário para confirmar a sua identidade.

Palavras-chave: Células T CD4 naïve; homeostasia das células T, CD31, IL-7, via de sinalização Wnt/TCF1

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List of Abbreviations

Akt	Protein Kinase B
APC	Adenomatous Polyposis Coli Protein
ART	Antiretroviral Treatment
Bcl	B-Cell Lymphoma
CCR	C-C Chemokine Receptor
CD	Cluster Of Differentiation
c-Myb	Cellular Myelocytomatosis Oncogene
CXCR	C-X-C Chemokine Receptor
DKK-1	Dickkopf-1
ERK	Extracellular Signal-Regulated Kinase
FOXO	Forkhead Box Transcription Factor
FOXP3	Transcription Factor Forkhead Box Protein 3
Frz	Frizzled
GATA3	Gata Binding Protein 3
GSK3	Glycogen Synthase Kinase 3
HIV-1	Human Immunodeficiency Virus-1
ICOS	Inducible Costimulator
IL	Interleukin
IL-2Rα	Interleukin 2 Receptor Alfa
IL-7Rα	Interleukin 7 Receptor Alfa
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
JAK	Janus Kinase
LEF1	Lymphoid Enhancer-Binding Factor 1
LRP	Lipoprotein-Receptor-Related Protein
MAPK	Mitogen-Activated Protein Kinase
MCL	Myeloid Cell Leukemia
MEK	Mitogen-Activated Protein Kinase Kinase
MFI	Median Fluorescence Intensity

MHC	Major Histocompatibility Complex
NAD	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer Cell
PBMC	Peripheral blood mononuclear cell
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PI3K	Phosphoinositide 3-Kinase
PTK	Protein-Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
RTE	Recent Thymic Emigrant
SHIP	Sh2-Containinginositol 5'-Phosphatase
SHP-2	Src Homology Region 2 (Sh2)-Containing Protein Tyrosine Phosphatase 2
sjTREC	Signal-Joint T- Cell Receptor Excision Circle
SLO	Secondary Lymphoid Organ
SOCS	Suppressor Of Cytokine Signalling
STAT	Signal Transducer and Activator of Transcription
T-ALL	T-Cell Acute Lymphoblastic Leukemia
TCF	T Cell Factor
Tconv	Conventional T Cell
TCR	T Cell Receptor
Tfh	Follicular Helper T Cells
Th	T Helper Cell
TMNC	Tonsillar mononuclear cell
Treg	Regulatory T Cell
Tscm	Stem Cell-Like Memory T Cell
UMAP	Uniform Manifold Approximation and Projection
Zap70	Zeta-Chain Associated Protein Kinase 70
γc	γ-chain

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Introduction

1.1. Human Immune Competence and the Importance of Maintaining a Healthy Naïve T cell compartment.

The human naïve T cell compartment is essential to maintain the immunocompetence of the individual throughout their lifetime. Naïve T cells are responsible for building immune responses against new antigens, generating, upon activation, the pool of memory-effector T cells. Their ability to respond to the vast potential of different antigens is due to their highly diverse T cell receptor (TCR) repertoire, established during T cell development in the thymus. This specific TCR is thus unique to each T cell and its clones, generated by division subsequent to their thymic generation[1].

Once in the periphery, mature naïve CD4+ T cells patrol the body, circulating between the blood and secondary lymphoid organs (SLOs), where antigens will be presented. It is also in the SLOs that mature human naïve CD4 T cells find survival and homeostatic signals that support their potentially long lifetime and slow turnover rate[2–4]. However, the naïve T cell compartment functions as a reservoir of specific immune cells against future potential pathogenic antigens, and the time required for a naïve T cell to encounter a specific antigen can vary, from mere minutes to years. While absolute numbers of the naïve compartment are kept, there is a need for the system to balance the survival of all potentially useful cells, whether it be the recently mature T cells that just left the thymus, also designated as recent thymic emigrants (RTEs), or cells that have been circulating in the periphery for longer[5]. Therefore, it is important to understand how the immune system supports and manages the heterogeneity of the naïve T cell compartment to maintain homeostasis.

The naïve T cell compartment is impacted on by physiological processes such as the recruitment of cells during immune responses or the reduction in thymic export that occurs with age. Additionally, other events such as disease or medical procedures can lead to important disturbances in the homeostasis of the naïve T cells, eventually leading to immunodeficiency. In cases of HIV infection, for example, people suffer from a striking decrease in absolute counts of naïve CD4 T cells, resulting from the overall activation and disruption of the whole T cell homeostasis occurring with the progression of the HIV-1 disease[6,7]. Importantly, when given antiretroviral treatment (ART), a higher frequency and absolute number of RTEs has been associated with better immune reconstitution of the patients and long-term prognosis and early treatment has been associated with better preservation of this compartment [8–11]. In the clinical setting, monitoring naïve CD4 T cell number, phenotype and function is thus useful to study disease progression or predict patients' successful recovery. For all the above, knowing how to manipulate the immune system in a way to promote the survival and proliferation of the naïve CD4 T cells while preserving their diversity is of the utmost importance to sustain immunocompetence.

Although once considered a homogenous and quiescence compartment of cells, it is now well established that naïve CD4 T cells are heterogeneous in terms of phenotype, function, and even, homeostatic responses. Therefore, by deepening our understanding of the naïve CD4 T cell functional and homeostatic heterogeneity and learning its modulation, we will be in a position to develop novel therapeutic interventions to either contribute to its reestablishment after disruption or prevent its erosion.

1.2. CD4 Naïve T cell compartment: Homeostasis and Heterogeneity.

The thymus handles the generation and establishment of the naïve T-cell pool from embryonic life and throughout life. However, in humans, and in contrast to mice, with the progressive loss of thymic output, the maintenance of the naïve compartment is sustained mainly through post-thymic peripheral proliferation (Figure 1)[12,13]. Indeed, naïve CD4 T cells undergo cell division, although at slow rates[2]. The main signals that promote naïve T cell peripheral proliferation and survival, while preserving their naïve phenotype, are TCR stimulation, by low-affinity self-peptides/MHC (major histocompatibility complex) interactions, and cytokines, mainly interleukin-7 (IL-7). Both signals are able to independently promote naïve T cell survival, however, it is not clear how they interact, being both signals widely available. While some suggest they may have a synergistic effect, other evidence states that tonic TCR stimulation initiates a dominant survival program, independent of IL-7-mediated survival[14,15]. Even though thymic output and post-thymic peripheral proliferation contribute together to sustain the naïve T cell compartment, it is only in the thymus that TCR assembly occurs. Thus, peripheral homeostasis is only able to maintain the already existing diversity of the TCR repertoire. As a consequence, older or thymectomized individuals, who rely deeply on the proliferation of peripheral T cells, are reported to have many alterations in the TCR repertoire[16–18].

On the other hand, naïve CD4 T cells can leave the compartment either by cell death or by activating and differentiating into memory-effector T cells (Figure 1). Out of the memory-effector compartment subsets, stem cell-like memory T cells (T_{scms}) show a particularly similar phenotype to the naïve T cells. This subset is a recently described memory population and, therefore, these cells have been included in older studies of human naïve T cells, as they express naïve markers such as CD45RA, CCR7 and CD27, being identifiable mainly by their expression of CD95[19,20]. Due to this and considering the unique proprieties of this population (further described below), their homeostatic properties and the processes that can regulate the generation of these cells out of naïve T cells have been an active research axis. The Wnt signalling pathway, (also referred in more detail below), has been highly regarded in this research topic. This pathway, known for its role in embryonic development, cell fate determination and stem cell maintenance, besides being employed in T_{scms} generation *in vitro*, has also been shown to modulate many other T cell processes. Part of its importance can be explained

by how it regulates TCF1 function, a transcription factor involved in T cell development and maintenance of T cell identity, function and differentiation[21–23].

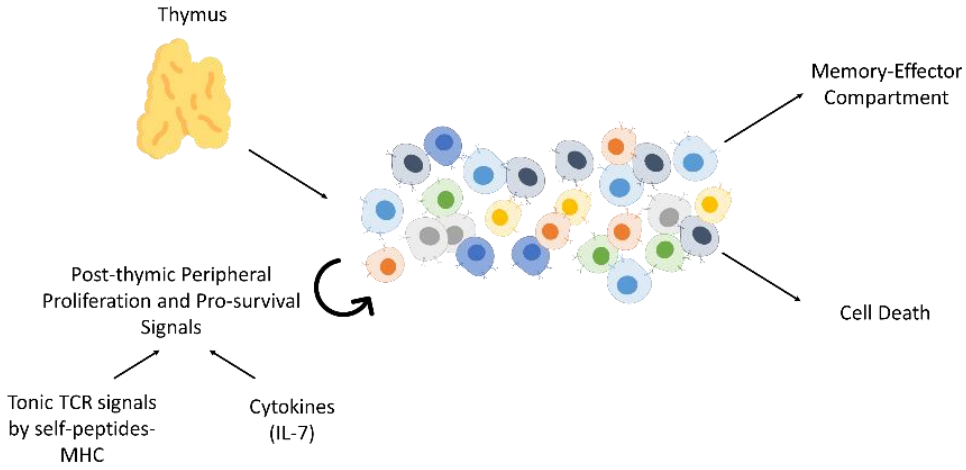


FIGURE 1: HOMEOSTASIS OF THE HUMAN NAÏVE CD4 T CELL COMPARTMENT. CD4 T cells originate from the thymus, from where they leave as RTEs once they are completely mature. In the periphery, this compartment of cells is sustained by pro-survival signals and slow proliferation rates induced by cytokines, mainly IL-7, and tonic TCR stimulation, promoted by the binding with self-peptides. On the other hand, the number of cells within the naïve CD4 T cell is also controlled by processes such as cell death or, upon an infection, T cell activation and differentiation into memory-effector CD4 T cells.

When we take into consideration the so-far known heterogeneity of the naïve CD4 T cell compartment, a new level of context to the homeostatic-related signalling is uncovered. In fact, within this compartment, it is possible to identify functionally distinct populations, such as naïve T_{regs} (regulatory T cells) and naïve T_{conv} (conventional T cells), and populations with distinct proliferative profiles. In this regard, CD31 has been pointed as a marker whose expression separates the naïve T cell pool in two subsets according to homeostatic proliferative profiles[3]. Moreover, although debatable, CD31 has been also used as a RTEs marker, since when leaving the thymus the vast majority of the mature CD4 naïve T cells express it[24]. Interestingly, the marker itself, CD31, is able to promote signalling, raising questions concerning a possible role for CD31 in the homeostasis of naïve CD4 T cells.

1.2.1 IL-7 signalling in naïve CD4 T cell homeostasis.

IL-7 has been pointed out as the most relevant cytokine for the homeostatic survival and proliferation of the naïve CD4 T cells in the periphery. In both animal models and human cell cultures, it has been shown that IL-7 is able to promote proliferation and survival within this compartment while maintaining the naïve phenotype[25–30]. In the physiological context, IL-7 is produced by stromal cells, of the epithelial and endothelial type, in several organs and tissues, including, the thymus and bone marrow (primary lymphoid organs) but also lymph nodes, lymphatic vessels, spleen and tonsils (secondary lymphoid organs)[31].

IL-7 is a member of the common γ-chain receptor (γc) family, which also includes other cytokines such as IL-2, IL-4, IL-9, IL-15, and IL-21. Its binding to the IL-7 receptor, constituted by the γc chain and the IL-7 receptor

alpha (IL-7Rα or CD127) chain, triggers a signalling cascade that leads to the upregulation of anti-apoptotic proteins and low levels of proliferation in naïve CD4 T cells[32,33]. The most relevant signalling pathways activated by IL-7 are the JAK/STAT and PI3K/AKT (see Figure 2). It is worth mentioning that MAPK/MEK/ERK signalling was also shown to be activated upon IL-7 stimulation in human T cell lines, however, it has not been implicated in IL-7-mediated cell survival and proliferation[30,34].

IL-7 receptor recruits and activates STAT5, after JAK1 and JAK3 phosphorylation, which, leads to its trafficking to the nucleus. Here, the activated STAT5 acts as a transcription factor and promotes the expression of pro-survival (Bcl-2 and MCL-1), proliferative (Cyclin D1), and negative feedback intermediator (SOCS, Suppressor of cytokine signalling) proteins[35–38]. The activation of the PI3K/Akt pathway is also important for T cell homeostasis and survival. Akt inhibits the GSK3/FOXO1/FOXO-3a complex, which, in turn, promotes Bcl-2 and GLUT1 expression, glucose uptake and down-regulates the expression of $p27^{kip1}$, a cell cycle inhibitor[34,39]. Akt is also able to phosphorylate and inhibit BAD, a Bcl-2 inhibitor[40]. In mice models, according to the levels of exposure to the cytokine, IL-7 can activate PI3K and STAT5 differently, activating different mechanisms to promote T cell survival. This has been suggested to have implications on T cell homeostasis and fitness[27,41].

Once stimulated with IL-7, T cells also go through negative feedback that down-regulates CD127 expression. At the post-translation level, CD127 is internalized and targeted for degradation by SOCS1[36,42]. At the transcriptional level, FOXO1, the transcription factor responsible for CD127 expression in naïve T cells, is inhibited by Akt[43]. This negative feedback has been described as an "altruistic" phenomenon because it limits the consumption of IL-7 per cell and, in turn, increases the number of cells exposed to the cytokine[44,45]. In the homeostatic context, this phenomenon supports the consideration of IL-7 as a broad, non-selective promoter of naïve CD4 T cell proliferation and survival.

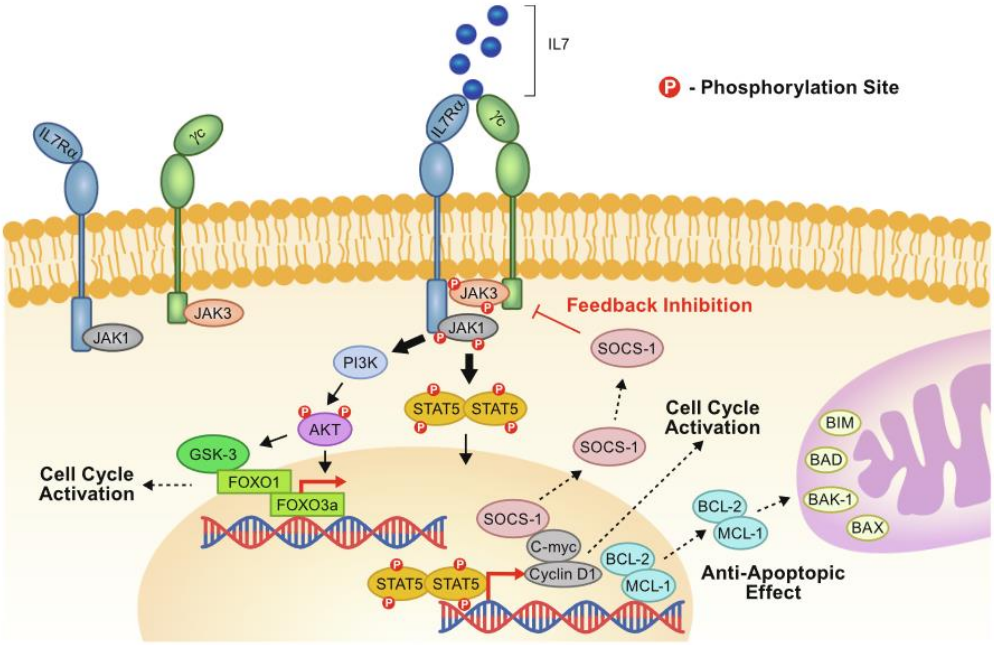


FIGURE 2: IL-7 SIGNALLING PATHWAYS IN T CELLS (RETRIEVED FROM WINER ET AL.)[33]. The Binding of IL-7 to its receptor subunits, IL7Rα and γc, and the consequential phosphorylation of the JAK1 and JAK3 molecules initiates a signalling cascade responsible for the pro-survival and pro-proliferation effects in naïve CD4 T cells. The IL-7 signalling pathway includes the PI3K/Akt signalling, which controls the transcriptional regulation by the complex GSK3/FOXO1/FOXO3, and the STAT5 pathway, which regulates the transcription of IL-7-related anti-apoptotic and pro-proliferative molecules.

1.2.2 Tonic TCR signalling in naïve CD4 T cell homeostasis.

Naïve T cells are also dependent on continuous stimulation of the TCR for survival. This stimulation consists of a low-level tonic stimulation created by low-affinity contact with self-peptides/MHC that promote pro-survival signals without inducing activation or cell division[46,47]. Furthermore, these signals are also important for naïve T cells to maintain self-tolerance in an environment of constant change, since TCR tuning allows for naïve T cells to adjust their activation threshold and, thus, diminish T cell auto and high-reactivity[48,49]. T cells transduce TCR signalling strength by promoting different signalling pathways. Weak TCR stimulation only triggers low-level phosphorylation of proximal targets of the TCR pathway, including TCRζ and Zap70, and, on the other side, up-regulates various negative regulators, including CD5 and SHP-1[50–52].

In opposition to IL-7 which promotes survival and proliferation of all cells expressing IL-7R, tonic TCR stimulation depends on the individual TCR specificity of the cell, creating a selection similar to what happens during T cell development[53]. According to Seddon and Zamoyska, depending on the compartment needs and individual responsiveness of the naïve T cells, TCR and IL-7 stimuli can work individually or synergize[14]. However, there is also evidence that TCR stimulation can antagonize IL-7 homeostatic response while promoting survival by alternative pathways[15]. Although somewhat contradictory evidence, if we take into consideration the heterogeneity of the naïve compartment, we have that certain subpopulations may have differential responses to TCR and IL-7 homeostatic stimuli.

1.2.3 TCF1 and its Wnt signalling-dependent and -independent functions in naïve CD4 T cell homeostasis.

T cell factor 1 (TCF1) is a transcription factor encoded by the gene *TCF7* and is mostly known as a downstream effector in canonical Wnt signalling (see Figure 3). In the steady state, the canonical Wnt pathway is constitutively inhibited. Indeed, β-catenin is maintained at low levels by a degradation complex that includes protein kinases, GSK-3β (glycogen synthase kinase) and APC (adenomatous polyposis coli) proteins. Once the Wnt protein binds to its receptor subunits, Frz and LRP5/6, it activates the pathway, the degradation complex is inhibited, allowing β-catenin to translocate to the nucleus. Here, β-catenin associates with transcription factors TCF1/LEF1, forming a complex that promotes chromatin accessibility and gene expression of Wnt-related genes[21,54–56]

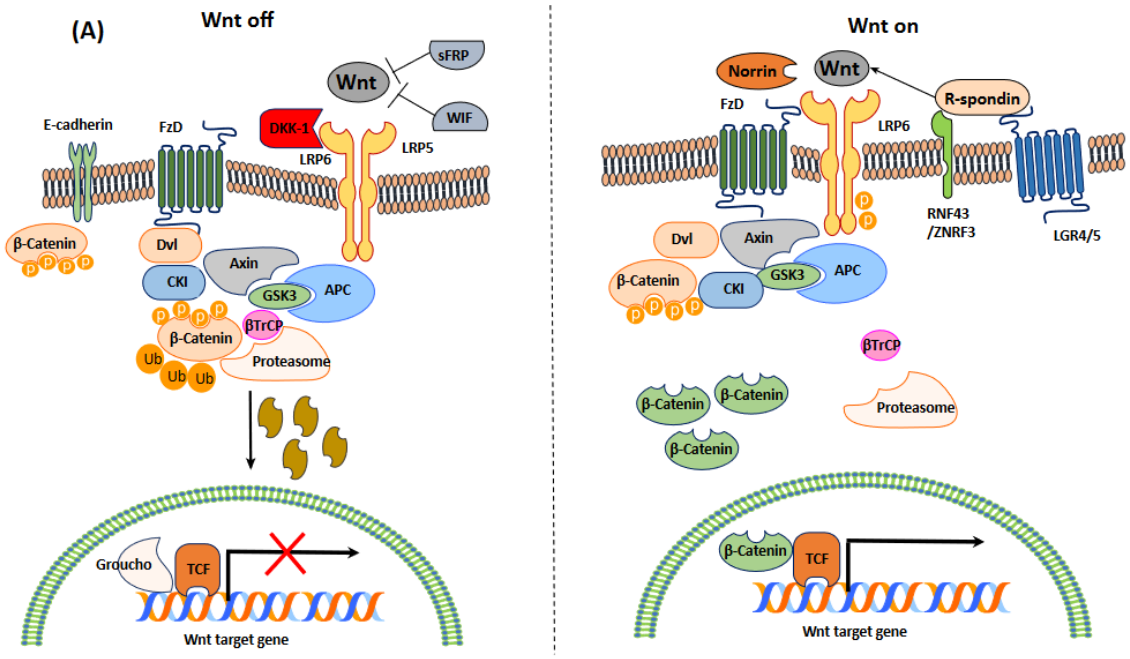


FIGURE 3 CANONICAL WNT SIGNALLING (RETRIEVED FROM CHAE AND BOTHWELL ET AL.)[56]. In steady state, the canonical WNT signalling is repressed (Wnt-OFF) by a complex responsible for targeting β-catenin, the main driver of this pathway, for degradation. Once Wnt binds to its receptor (Wnt-ON) it activates the pathway which leads an accumulation of β-catenin in the cytoplasm and its translocation to the nucleus, where it regulates the transcription of Wnt-related genes.

Due to alternative splicing, though, TCF1 has different-sized isoforms with repercussions on the protein's binding and function: while the full-length protein contains a β-catenin-biding domain, the shorter isoforms lack the same domain and, therefore, cannot interact with β-catenin. Since this fact is sometimes left out of consideration in published studies, it's difficult to pinpoint whether TCF1 roles in T cell processes are Wnt signalling dependent or independent. However, the studies that consider Wnt as an important player show that TCF1 isoform types show distinct but cooperative functions[21,57,58]. Nonetheless, both TCF1 and Wnt signalling are reported to be involved in T cell development in the thymus, T cell migration and even CD4 T cell differentiation into T helper subsets[21,22,55,57–60]. For example, during T cell development in the thymus, whereas β-catenin-interacting TCF1 isoforms are necessary for thymocyte survival, maintaining thymic output, the shorter isoforms are responsible for establishing the epigenetic identity of T cells and supporting the progress through the maturation steps.

Wnt signalling and TCF1 are also associated with the maintenance of the naïve T cell phenotype. Studies focusing on naïve T cell profiling show that TCF1 is important to maintain the less differentiated phenotype of naïve T cells[61,62]. Moreover, upon activation, this pathway is down-regulated and binding sites become inaccessible[63–66]. In *in vitro* modulation, it has been reported that activation of Wnt signalling arrests the naïve T cell phenotype while also inhibiting T cell expansion and effector functions[67–69].

Although these studies establish an important role of Wnt/TCF1 in the maintenance of the naïve T cell "stemness", it is less clear if they are relevant for mature naïve T cell survival and homeostatic proliferation in the periphery. There are only a few studies that show some level of interaction between IL-7 and Wnt/TCF1 in T cells. Langhammer et al. showed, in T-ALL cell lines, that PI3K/Akt activation leads to accumulation of β-catenin in the cytoplasm, though translocation of the protein to the nucleus did not occur[70]. Additionally, while studying thymocyte development in mouse models, Yu et al. reported that β-catenin enhances IL-7 signalling response, while IL-7 signalling inhibits TCF-1 and LEF-1 signalling[56,71,72]. However, these studies in T cell precursors may or may not be translatable to mature human naïve T cells.

1.2.4 Wnt signalling and T_{scms} generation.

T_{scms} are a recently described memory T cell subset with stem cell-like proprieties, including self-renewal and the capacity to generate all memory-effector T cell subsets *in vitro*. According to transcriptome analysis, they are the most similar memory subset to naïve T cells, thus explaining their also alike phenotype. Due to these proprieties, they show a high potential to be used in T cell-based therapies, as already reviewed[23,73].

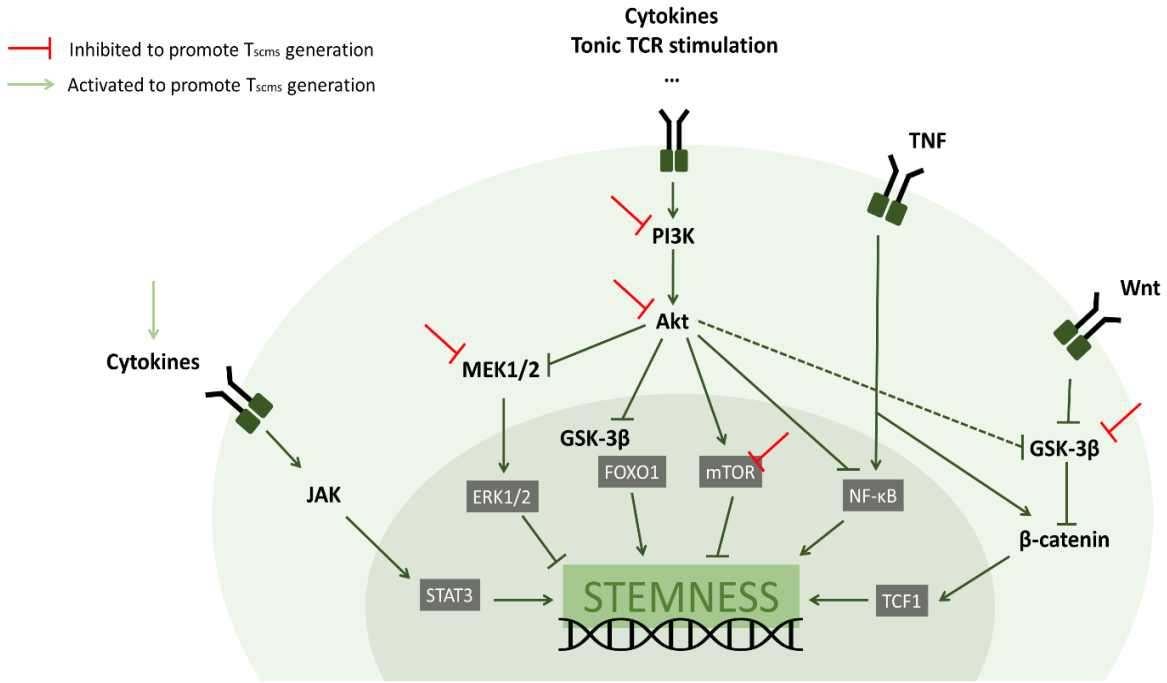


FIGURE 4: POSSIBLE SIGNALLING PATHWAYS INVOLVED IN T_{SCMS} GENERATION (ADAPTED FROM WANG ET AL.) [23] Based on previously published studies, many signalling pathways have been reported to be involved in the generation of T_{scms}. Indeed, the pharmacological targeting of molecules involved in these pathways, such as GSK3 in the Wnt pathway or the PI3K/Akt/mTOR pathways, have shown to induce transcription programs associated with this memory subset.

Many signalling pathways have been shown to induce T_{scms} generation (see Figure 4), including Wnt/β-catenin, PI3K/Akt, JAK/STAT and MAPK pathways (reviewed by Wang *et al.*)[23]. However, most studies have been focused on CD8 T_{scms} only. Here, the focus will be on the canonical Wnt signalling, the only pathway that has been shown to promote specifically CD4 T_{scms} generation[55]. Nevertheless, it is important to have in mind the potential crosstalk between all these pathways and their respective potential consequences in the T_{scms} generation.

Kared et al. demonstrated that is possible to induce CD4 T_{scms} with Wnt activation, using a GSK-3β inhibitor, and TCR stimulation. Furthermore, they also show that aging-associated dysregulation of Wnt/β-catenin signalling and the systemic increase of DKK-1 (Dickkopf-1), a natural inhibitor of Wnt, are accompanied by impairments of T_{scms} homeostasis, including their proliferative response to IL-7[55].

Although most protocols performed to induce T_{scms} in vitro relied on pharmacological modulation of signalling pathways (see Figure 4), Cieri et al. showed that TCR stimulation and IL-15 and IL-7 were sufficient to generate this T cell subset[74]. It is possible that IL-7 (and IL-15) promotes the generation of T_{scms} upon TCR activation by activating Wnt signalling or another pathway that promotes stemness. However, it remains unclear whether the molecular pathways by which TCR stimulation and cytokines, especially IL-7, interact to originate T_{scms} from naïve T cells and whether these pathways can be manipulated to allow the expansion of naïve CD4 T cells or subsets of these.

1.2.5 Heterogeneity in the human Naïve CD4 T cell compartment and homeostasis.

As has been pointed out above, novel studies focusing on the naïve CD4 T cells have been reporting a new level of heterogeneity of this compartment, both in phenotype, function and, interestingly, homeostatic maintenance[3,5].

Within the compartment, it is already well-established the existence of both naïve T_{convs} and, the regulatory T cell subset, T_{regs}. Interestingly, these two subsets also show different homeostatic needs. Indeed, our lab has reported that, in thymectomized adults, the maintenance of naïve regulatory T cell pool was less dependent on thymic output, when compared with the conventional naïve T cells. Although when stimulated *in vitro* with IL-7 both subsets have comparable responses, when evaluating the IL-7-response readouts *ex-vivo,* T_{regs} show higher levels of Bcl-2, pSTAT5 and Ki-67, indicating that they are continuously responding to IL-7[29].

Other subsets have been identified within the naïve CD4 T cell compartment. Besides the previously mentioned T_{scms}, it was also identified a subset of very recently activated T cells, that express CXCR3 and CCR4 and are speculated to be in a “resting state” before acquiring a full memory phenotype. Although this subset expresses many naïve markers, including CD45RA, they show functional characteristics of memory T cells, such as the ability to produce effector cytokines[75]. In juxtaposition, RTEs are an important subset with low to no

exposure to peripherical homeostatic stimuli since they recently left the thymus. Thus, this subset may shed light on changes that occur when mature naïve CD4 T cells respond to TCR and IL-7-mediated homeostatic signals. As previously mentioned, CD31 is a commonly used marker to identify RTEs, however, better markers have been proposed. For example, the expression of PTK7, a receptor tyrosine kinase, is also associated with recent thymic emigrants. In fact, in thymectomized individuals, this marker rapidly declines in the naïve CD4 T cell compartment and, *in vitro* experiments, PTK7 is downregulated upon cell division[5,76,77].

The focus of this project, however, will be on the subsets within naïve CD4 T cells that can be distinguished according to the expression of CD31[17,30,78]. Although they don't reveal distinct functional phenotypes, they are shown to have different proliferative histories (further described below). CD31 is a marker shown to modulate many signalling pathways on T cells and it has been considered an immune inhibitory receptor[79]. However, many questions regarding its role in naïve CD4 T cell homeostasis remain unanswered.

1.3. CD31 (PECAM-1).

1.3.1. Overview of Structure, Ligands and Signalling.

CD31 or PECAM-1 (platelet endothelial cell adhesion molecule-1) is part of the immunoglobulin (Ig) gene superfamily. It has six extracellular Ig-like domains (D1 to D6) and, within its cytoplasmic domain, it has two immunoreceptor tyrosine-based inhibition motifs (ITIMs)[78,80]. CD31 is expressed in immune cells, namely, T cells, mast cells, NK cells and monocytes, but is also expressed in platelets and endothelial cells (ECs), where its role as a cell adhesion molecule has been thoroughly studied. Its main ligand it's oneself, but it also has heterophilic interaction with various ligands, most importantly CD38 (ADP-ribosyl cyclase), but also, integrin αvβ3 and CD177 [78,80,81].

ITIM-bearing receptors, such as CD31, are known to regulate immunoreceptor tyrosine-based activation motifs (ITAMs)-containing receptors, although their inhibitory capacity is not restricted to this class of receptors[79]. Through the ITIM domains, they are able to recruit protein tyrosine phosphatases(PTPs), such as SHIP, SHP-1 and SHP-2, which leads to the activation of different signalling pathways[81,82]. Several PTPs-dependent CD31 signalling events have been described, including platelet aggregation, leucocyte transmigration and mobility, the inhibition of TCR-mediated signalling and regulation of β-catenin activity[81,83–86]. Newton-Nash and Newman had shown that upon activation of Jurkat cells, the oligomerization of CD31 interferes with the TCR-mediated calcium mobilization[83,87]. However, the effects of CD31 inhibition were only on a short term which hints that CD31 delays the activation of T cells but does not hamper the magnitude of activation, once they are activated[83]. On the regulation of β-catenin activity, Biswas et al. has shown in endothelial cells that upon CD31 signalling activation, the recruited SHP-2 dephosphorylates β-catenin, activating it and allowing it to translocate to the nucleus. Moreover, CD31 also hinders GSK-3β inhibitory activity, possibly by a PI3K/Akt dependent pathway[85,86].

Due to its relevance in many T cell functions and the link to signalling pathways involved in T cell homeostasis, CD31 has been studied in the context of CD4 T cell homeostasis.

1.3.2. CD31 expression in the naïve CD4 T cell compartment.

CD31 expression is known to vary throughout the stages of T cell development and between T cell functional subsets. Once they leave the thymus, mature naïve T cells express CD31 and, upon activation and differentiation to effector T cells, they lose it. However, a key difference exists between CD4 and CD8 T cells: while CD4 T cells do not re-express CD31 after activation, memory CD8 T cells do[30,88,24].

Within the naïve CD4 T cell compartment, it is possible to distinguish two subsets with different homeostatic proliferative profiles, according to CD31 expression. In fact, CD31+ CD4 T cells have higher sjTREC content and longer telomere length than the $CD31^{neg}$ subset, hinting that CD31+ have gone through fewer rounds of cell division[89]. Additionally, work from our lab has reported that although both subsets are able to respond to IL-7, proliferation is only induced in the CD31+ CD4 naïve T cells[30]. Mold et al. builds on this observation by reporting that CD31 expression is positively correlated with the basal level of NF-κB phosphorylation, which, in turn, is essential for IL-7-driven homeostatic proliferation[2]. In contrast, the $CD31^{neg}$ subset is thought to rely more on the TCR tonic signals for survival, since they lose the inhibitory activity of CD31. In fact, CD31 is able to attenuate the TCR-mediated signalling, in a SHP-2-dependent way[82,90]. Moreover, when studied in the context of T cell activation, in expressing cells, CD31 is thought to be cleaved due to the TCR stimulation[90,91]. The rescue of the CD31 signalling in the CD31-truncated cells, is reported to inhibit the TCR-promoted T cell activation[90,92].

Summarizing, the established consensus is that once naïve CD4 T cells leave the thymus, they express CD31, thus RTEs are included in this subset, and their proliferation and survival are driven by IL-7. However, as they go through rounds of cell division and receive TCR stimulation, they lose the expression of CD31 and depend on TCR-driven survival signals[90].

1.4. Aims of this project.

To be able to modulate the naïve T cell compartment in health and disease, first, it is vital to understand the full extent of naïve T cell heterogeneity and which signals can be used to maintain or regenerate the pool of cells. Given the previous studies on the function of CD31 on T cell activation, we hypothesized that the differential expression of this molecule within the naïve CD4 T cell pool may be related to a functional role related to the distinct homeostatic requirements of the subsets and, therefore, to different responses to homeostatic stimuli. Additionally, recent findings related to the expansion and homeostasis of the T_{scms} compartment raise relevant questions on the possibility to intervene in naïve CD4 T cell homeostasis by Wnt signalling or GSK3 modulation, in relation to, or not, with the CD31-defined phenotypes.

As the first aim of the project, we proposed to search for links between Wnt or TCF1 axis, CD31 expression and IL-7 signalling, characterizing, with the use of multiparameter flow cytometry, the expression of relevant markers in the human naïve T cell compartment. Paired samples of blood and tonsil of children who went through tonsillectomy were analysed in order to investigate changes in different sites concerning homeostatic signals.

In the second aim of the project, we deepened our understanding of how differently CD31-defined naïve CD4 T cell subsets respond to homeostatic stimuli. Included in this, we investigated the impact of CD31 modulation in naïve CD4 response to homeostatic stimuli. For this purpose, we cultured sorted human naïve CD4 T cells in the presence of TCR and/or IL-7 stimulation, with different CD31 agonist or antagonist modulators, in established short time points.

Finally, we proposed to investigate a possible crosstalk between IL7 and Wnt/TCF1 signals and whether CD31 subsets respond differently. Using the same strategy as in the second aim, we cultured sorted human naïve CD4 T cells, in the presence of TCR and/or IL-7 stimulation, with agonist or antagonist modulators of the Wnt/TCF1 signalling, in established short and long time points.

Material and Methods

2.1. Human Sample Collection.

2.1.1. Tonsil and Blood-Paired Samples.

Human tonsils and paired blood of children who underwent tonsillectomy were provided by the ENT Department of the Hospital Santa Maria with the written consent of parents or legal representatives of said children. The experiments comply with the ethical principles under the European Commission Directive 2004/23/EC and Portuguese Law No 12/2009. The samples are pseudo-anonymized (code will be used for all experiments) to protect personal data.

Human tonsils were collected in 50mL falcons with 15mL of Tonsils' Media (RPMI-1640 medium (RPMI) supplemented with 10% Foetal bovine serum (FBS), 1% L-glutamine, 1,5% Pen-Strep, 1x Sodium Pyruvate, 1x MEM Non-Essential Amino Acids Solution and 0,1% Gentamicin (all reagents from Gibco)). The blood samples were collected into 10mL Glass Sodium Heparin Tubes (BD Vacutainer®).

2.1.2. Buffy Coats.

Buffy Coats of healthy donors, female, under the age of 45 years old, with unspecified blood type, were obtained under a protocol with Instituto Português do Sangue e da Transplantação (IPST). The experiments comply with the ethical principles under the European Commission Directive 2004/23/EC and Portuguese Law No 12/2009.

2.2. Human Samples Processing and Cell Isolation.

2.2.1. Tonsil and Blood-Paired Samples.

Tonsils were placed in sterile culture dishes with RPMI supplemented with 2% FBS (RPMI 2%FBS) to proceed with dissection, mechanic disruption and cell suspension filtration using a 70 µm membrane cell strainer. Tonsillar cells were then collected and resuspended into a 50mL falcon with RPMI 2% FBS. The blood was diluted in a 1:1 ratio with Phosphate-buffered saline 1x solution (PBS 1x; Sigma-Aldrich).

Tonsillar mononuclear cells (TMNCs) and Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient (Ficoll-Paque Plus; GE Healthcare) in a 2:1 ratio of collected cells from the samples and Ficoll-Paque solution, respectively, in a 50mL falcon. After the centrifugation of 20 minutes at 2000 rpm, the rings of mononuclear cells were collected into universal tubes and washed twice with PBS 1x (centrifugation of 10 minutes at 1600rpms). Finally, the cells were counted with a Trypan Blue solution (Sigma) in a Neubauer chamber.

2.2.2. Buffy Coats.

About 40mL of each collected Buffy Coat was retrieved from the blood bags into T75 Flasks and diluted in a 1:2 ratio with PBS 1x. The isolation of the PBMCs proceeded with a Ficoll density gradient in a 2:1 ratio and

centrifugation of 20 minutes at 2000rpms. The cells from the resulting ring were collected into a new universal tube and washed twice with PBS 1x (centrifugation of 10 minutes 1600rpms). Erythrocytes were lysed by adding, to the cell pellet, 5mL of 1x Red Blood Cell (RBC) Lysis Buffer (Invitrogen, eBioscience™) for 10 minutes at room temperature (RT). Finally, the cells were washed once with PBS 1x (centrifugation of 10 minutes at 1600rpms) and counted with Trypan Blue solution (Sigma) in a Neubauer chamber.

2.3. Cell Sorting of CD4 Naïve CD4 T cells.

Buffy Coat's isolated PBMCs were used to sort human CD4 Naïve T_{convs} by negative selection using the MojoSort Human Naïve CD4 T cell Isolation kit (BioLegend), according to the manufacturer's protocol. This kit includes an anti-CD25 antibody and, therefore, it also excludes a significant part of the human CD4 naïve Tregs. Firstly, the PBMCs were washed with MojoSort buffer (PBS 1x with 0,5% BSA and 2mM EDTA; centrifugation 5 minutes at 1400 rpm), filtered with a 70 µm cell strainer, and resuspended with MojoSort buffer to adjust the cell concentration to 1 x 10^8 cells/mL. About 2,2 x 10^8 cells were aliquot to a FACS tube, the Biotin-Antibody Cocktail was added (10uL of cocktail/10^7 cells) and incubated for 15 minutes on ice. Then, the Streptavidin Nanobeads (10uL of beads/10^7 cells) were also added and incubated for another 15 minutes on ice. The FACS tube was placed on the magnet for 5 minutes and, afterwards, the enriched cell suspension was poured into a universal tube. This enrichment step was done twice to obtain a higher number of cells. The sorted cells were washed once with MojoSort (centrifugation 5 minutes, 1600 rpm), counted with Trypan Blue, and their concentration was adjusted to the needed one for the following cell cultures (see 2.4) with Culture Media (RMPI supplemented with 10% AB serum, 1% L-glutamine, 1% Pen-Strep, 1x Sodium Pyruvate, 1x MEM Non-Essential Amino Acids Solution and 0,1% Gentamicin (all reagents from Gibco)).

2.4. Cell Culture, Stimulation and Modulation Conditions.

2.4.1. Optimization of CD31 and Wnt Modulators' Concentration.

CD4 Naïve T_{convs} sorted with the MojoSort Magnetic sorting kit were cultured in 96 well plates at 10.000 cells/µL per well. At a concentration of 1:8 beads to cells ratio (DynaBeads Human T-activator CD3/CD28 (Gibco)) to promote TCR stimulation, several CD31 modulators at serial dilutions were added separately to the wells: anti-CD31 primary antibody, with blocking proprieties, (40, 20, 10 and 5 µg/mL; abcam, clone JC/70A), recombinant human CD31 peptide (100, 50, 25, 12,5 µg/mL; PeprotTech) and an SHP-2 inhibitor, SHP099, (100, 50, 25, 12,5 µM; Focus Biomolecules). As a control, cells were also cultured, with or without TCR stimulation at the same ratio, with the respective controls at the highest concentrations of each modulator: 40 µg/mL of isotype control (BioLegend) for the antibody and DMSO with the same added volume as SHP099. The cells were incubated for 3 hours at 37ºC.

Additionally, CD4 Naïve T_{convs} were cultured for a longer time with IL-7 (10ng/ml; PeproTech) and/or 1:32 beads to cells ratio and modulated with serial dilutions of Wnt signalling modulators. For activation of the signalling pathway, it was used CHIR99021 (Tocris), a GSK3 inhibitor, at 6, 3, 1,5 and 0,75 µM and, for inhibition, a natural inhibitor, DKK-1 (PeproTech), at 100, 50, 25 and 12,5 ng/mL. The controls were the same volume of DMSO as the highest concentration of CHIR99021 and negative control for DKK-1, in the same concentration of IL-7 and/or TCR stimulation. The cells were incubated for 3 days at 37ºC.

2.4.2. CD31 and Wnt signalling modulation in *in vitro* culture with homeostatic stimulation.

Sorted CD4 Naïve T_{convs} were cultured in 96 well plates at 20.000 cells/µL per well, with or without 1:8R stimulation and/or IL-7 (10ng/ml). The cells were modulated with anti-CD31 blocking antibody (50µg/mL), recombinant human CD31 peptide (25µg/mL), SHP099 (50µM), DKK-1 (50ng/mL) and CHIR99021 (3µM). As a control, it was used isotype control (50µg/mL) for the antibody, and it was added the same volume of DMSO as SHP099 and CHIR99021 for their control. The cells were incubated for 3 hours at 37ºC.

For the longest time point, cells were cultured with IL-7 (10ng/ml), IL-2 (20U/mL; PeproTech) or only culture media. Additionally, they were modulated individually with DKK-1 (50ng/mL), CHIR99021 (3µM) and respective controls. The cells were incubated for 6 days at 37ºC with replenishing of the conditions on day 3.

2.5. Flow Cytometry Staining and Acquisition.

2.5.1 Cell Surface and Intracellular Staining.

To follow with the staining, cells were transferred into wells in 96-well plates and washed with 100 µL of 1x PBS (centrifugation at 2000 rpm, 2 minutes). After removing the supernatant, the cells were incubated with the mix of surface antibodies (see tables 2, 3 and 5-8 for more details), Viability Dye (see tables 2, 3 and 5-8 for more details) and Human FcR Block Reagent (dilution 1/75, Miltenyi Biotec) for a final volume of 25 µL and incubated for 25 minutes at RT. After the incubation, cells were washed with 150 µL of 1x PBS and fixed 100µL with Fix Perm solution (solution prepared with ¼ of Fixation/Permeabilization Concentrate and ¾ of Fixation/Perm Diluent (both from Invitrogen)) for 30 minutes at 4ºC. Following fixation, the cells were washed twice, once with 100µL FACS buffer (PBS 1x with 2% FBS) and the other with 150µL Perm Buffer 1x (Permeabilization Buffer 10x (Invitrogen) diluted with distilled water). The fixed cells were then incubated with a mix of intracellular antibodies (see tables 2, 3 and 5-8 for more details) for 30 minutes at 4ºC. Lastly, the cells were washed with 150µL Perm Buffer 1x, resuspended in 200µL FACS buffer and transferred to a FACS tube for acquisition.

All samples were acquired in the spectral flow cytometer Cytek Aurora (Cytek Biosciences), using the SpectroFlo software.

2.5.2. Antibody Panels Used.

TABLE 1: PAIRED TONSIL AND BLOOD STAINING PANEL

Antibody	Fluorochrome	Clone	Dilution	Brand Manufacturer
Flow Cytometry Surface Staining Panel				
CD31	BV 421	MEC 78.2	1:50	BD
CD4	BV 480	OKT4	1:150	BD
HLA-DR	Spark Violet 538	L243	1:120	BioLegend
CD45RO	BV 570	UCHL1	1:100	BioLegend
PD-1	BV 605	EH12.2H7	1:150	BioLegend
CD45RA	BV 650	HI100	1:100	BioLegend
CCR7	BV 711	G043H7	1:30	BioLegend
CD31	BV 785	WM59	1:200	BioLegend
CD45	Alexa Flour 532	HI30	1:100	Invitrogen
CXCR5	PE-Dazzle 594	J252D4	1:100	BioLegend
CD25	PE-Cy5	BC96	1:120	BioLegend
CD5	PerCP	UCHT2	1:50	BioLegend
CD38	PE-Fire 700	S17015A	1:150	BioLegend
CD27	PerCP-eFlour 710	O323	1:50	eBioscience
CD95	PE-Cy7	DX2	1:150	eBioscience
CXCR3	PE-Fire 810	G025H7	1:50	BioLegend
CD69	Spark NIR 685	FN50	1:200	BioLegend
ICOS	Alexa Flour 700	C398.4A	1:50	BioLegend
CD127	APC-eFlour 780	eBioRDR5	1:75	eBioscience
CD3	APC-Fire 810	SK7	1:150	BioLegend
Flow Cytometry Intracellular Staining Panel				
FOXP3	eFlour 450	PCH101	1:50	eBioscience
Bcl-2	FITC	124	1:50	Dako
TCF-1	PE	7F11A10	1:100	BioLegend
Ki-67	PerCP-Cy5.5	B56	1:60	BD
β Catenin	APC	12F7	1:50	BioLegend
Flow Cytometry Viability Dye				
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit			1:100	Invitrogen

TABLE 2: CD31 MODULATION OPTIMIZATION (3 HOURS CULTURE) STAINING PANEL

Antibody	Fluorochrome	Clone	Dilution	Brand Manufacturer
Flow Cytometry Surface Staining Panel				
CD31	BV 421	MEC 78.2	1:50	BD
CD4	BV 480	OKT4	1:150	BD
ICOS (CD278)	BV 510	C398.4A	1:120	BioLegend
CD45RO	BV 570	UCHL1	1:100	BioLegend
CD45RA	BV 650	HI100	1:100	BioLegend
CD40L[1]	BV 711	24-31	1:400	BioLegend
CD31	BV 785	WM59	1:200	BioLegend
CCR7	FITC	150503	1:30	R&D Systems

CD25	PE-Cy5	BC96	1:120	BioLegend
CD5	PerCP	UCHT2	1:50	BioLegend
CD38	PE-Fire 700	S17015A	1:150	BioLegend
CD27	PerCP-eFlour 710	O323	1:50	eBioscience
CD95	PE-Cy7	DX2	1:150	eBioscience
CXCR3	PE-Fire 810	G025H7	1:50	BioLegend
CD122	APC	TU27	1:75	BioLegend
CD69	Spark NIR 685	FN50	1:200	BioLegend
CD127	Alexa Flour 700	A019D5	1:43	BioLegend
CD3	APC-Fire 810	SK7	1:150	BioLegend
Flow Cytometry Intracellular Staining Panel				
FOXP3	eFlour 450	PCH101	1:50	eBioscience
TCF-1	PE	7F11A10	1:100	BioLegend
Ki-67	PerCP-Cy5.5	B56	1:60	BD
Flow Cytometry Viability Dye				
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit			1:100	Invitrogen

TABLE 3: WNT MODULATORS OPTIMIZATION (3 DAYS CULTURE) STAINING PANEL

Antibody	Fluorochrome	Clone	Dilution	Brand Manufacturer
Flow Cytometry Surface Staining Panel				
CD31	BV 421	MEC 78.2	1:50	BD
CD4	BV 480	OKT4	1:150	BD
ICOS (CD278)	BV 510	C398.4A	1:120	BioLegend
CD45RO	BV 570	UCHL1	1:100	BioLegend
CD45RA	BV 650	HI100	1:100	BioLegend
CCR7	BV 711	G043H7	1:30	BioLegend
CD31	BV 785	WM59	1:200	BioLegend
CD25	PE-Cy5	BC96	1:120	BioLegend
CD5	PerCP	UCHT2	1:50	BioLegend
CD38	PE-Fire 700	S17015A	1:150	BioLegend
CD27	PerCP-eFlour 710	O323	1:50	eBioscience
CD95	PE-Cy7	DX2	1:150	eBioscience
CXCR3	PE-Fire 810	G025H7	1:50	BioLegend
CD69	Spark NIR 685	FN50	1:200	BioLegend
CD127	Alexa Flour 700	A019D5	1:43	BioLegend
CD3	APC-Fire 810	SK7	1:150	BioLegend
Flow Cytometry Intracellular Staining Panel				
FOXP3	eFlour 450	PCH101	1:50	eBioscience
Bcl-2	FITC	124	1:50	Dako
TCF-1	PE	7F11A10	1:100	BioLegend
Ki-67	PerCP-Cy5.5	B56	1:60	BD
β Catenin	APC	12F7	1:50	BioLegend
Flow Cytometry Viability Dye				
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit			1:100	Invitrogen

TABLE 4: CD31 AND WNT SIGNALLING MODULATION (3 HOURS CULTURE) STAINING PANEL

Antibody	Fluorochrome	Clone	Dilution	Brand Manufacturer
Flow Cytometry Surface Staining Panel				
CD39	BV 421	A1	1:100	BioLegend
CD4	BV 480	OKT4	1:150	BD
ICOS (CD278)	BV 510	C398.4A	1:120	BioLegend
CD45RO	BV 570	UCHL1	1:100	BioLegend
CD45RA	BV 650	HI100	1:100	BioLegend
CD40L[1]	BV 711	24-31	1:400	BioLegend
CD31	BV 785	WM59	1:200	BioLegend
CD25	PE-Cy5	BC96	1:120	BioLegend
CD38	PE-Fire 700	S17015A	1:150	BioLegend
CD95	PE-Cy7	DX2	1:150	eBioscience
CXCR3	PE-Fire 810	G025H7	1:50	BioLegend
CD69	Spark NIR 685	FN50	1:200	BioLegend
CD127	Alexa Flour 700	A019D5	1:43	BioLegend
CD3	APC-Fire 810	SK7	1:150	BioLegend
Flow Cytometry Intracellular Staining Panel				
FOXP3	eFlour 450	PCH101	1:50	eBioscience
Bcl-2	FITC	124	1:50	Dako
TCF-1	PE	7F11A10	1:100	BioLegend
GATA3	PE-eFlour 610	TWAJ	1:25	Invitrogen
β Catenin	APC	12F7	1:50	BioLegend
Flow Cytometry Viability Dye				
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit			1:100	Invitrogen

TABLE 5: WNT SIGNALLING MODULATION EXPERIMENT (7 DAYS CULTURE) STAINING PANEL

Antibody	Fluorochrome	Clone	Dilution	Brand Manufacturer
Flow Cytometry Surface Staining Panel				
CD39	BV 421	A1	1:100	BioLegend
CD4	BV 480	OKT4	1:150	BD
ICOS (CD278)	BV 510	C398.4A	1:120	BioLegend
CD45RO	BV 570	UCHL1	1:100	BioLegend
CD45RA	BV 650	HI100	1:100	BioLegend
CD95	BV 711	DX2	1:100	BioLegend
CD31	BV 785	WM59	1:200	BioLegend
CD25	PE-Cy5	BC96	1:120	BioLegend
CD38	PE-Fire 700	S17015A	1:150	BioLegend
CXCR3	PE-Fire 810	G025H7	1:50	BioLegend
CD69	Spark NIR 685	FN50	1:200	BioLegend

[1] These antibodies were added directly in the culture wells when preparing the experiments conditions for a better staining as study by Koguchi et al.[93]

CD127	Alexa Flour 700	A019D5	1:43	BioLegend
CD3	APC-Fire 810	SK7	1:150	BioLegend
Flow Cytometry Intracellular Staining Panel				
FOXP3	eFlour 450	PCH101	1:50	eBioscience
Bcl-2	FITC	124	1:50	Dako
TCF-1	PE	7F11A10	1:100	BioLegend
GATA3	PE-eFlour 610	TWAJ	1:25	Invitrogen
Ki-67	PerCP-Cy5.5	B56	1:60	BD
T-bet	PE-Cy7	eBio4B10	1:50	eBioscience
β Catenin	APC	12F7	1:50	BioLegend
Flow Cytometry Viability Dye				
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit			1:100	Invitrogen

2.6. Supervised and Unsupervised Analysis of Flow Cytometry Data.

All compensations and supervised analysis of the flow cytometry data were performed using FlowJo v10.7 software (FlowJo LLC, USA).

For the unsupervised *ex vivo* characterization of the Human Naïve CD4 T cell compartment, the acquired samples were gated on CD45+CD3+CD4+CD45RA+CD45RO-CCR7+CD27+ to proceed with Uniform Manifold Approximation and Projection (UMAP) on FCS Express 7 Research Edition (De Novo Software, USA).

2.7. Graphical and Statistical Analysis.

Graphs and respective statistical analyses were all generated using GraphPad Prism (version 8.4.3). The majority of the statistical significance was calculated with either Paired T-tests when comparing two sets of observations, or two-way ANOVA with Sidak's or Tukey´s multiple comparison tests (according to what was recommended) when assessing the influence of two independent variables on one dependent variable, both tests with matched values according to the sample's donor. The family-wise significance and confidence level were set at 0.05 (95% confidence interval). The results presented follow the GraphPad p-value style (ns, $p>0.05$; *, $p\leq0.05$; **, $p\leq0.01$; ***, $p\leq0.001$; ****, $p\leq0.0001$).

Results

3. CD31 subsets within human naïve CD4 T cells in paired Tonsil and Blood samples.

To characterize the CD31 subsets within the naïve CD4 T cell compartment and gather clues in order to better understand the role of CD31, paired samples of blood and tonsil (n=4, age: 4 to 6 years old), were collected and processed for flow cytometry acquisition and analysis, using the antibody panel shown on Table 1. The paired samples allow us to establish the differences and create a parallel between the naïve CD4 T cells in the periphery (blood) and lymphoid tissue, akin to SLOs (tonsils), where they are exposed and respond to the homeostatic signals[31]. The main populations studied were identified according to the gating strategy illustrated in Figure 5 and Supplemental Figure 1. Here, we established two possible definitions of naïve CD4 T cells: "Broad" Naïve CD4 T cells (CD45+CD3+CD4+CD45RO-CD45RA+CCR7+CD27+), which include the stem-cell memory CD4 T cells, identified by the expression of CD95, and "True" Naïve CD4 T cells, which do not include T_{scms} (CD45+CD3+CD4+CD45RO-CD45RA+CCR7+CD27+CD95-). Within the "True" Naïve CD4 T cells, conventional (FOXP3-) and regulatory (FOXP3+) CD4 T cells were gated according to the expression of FOXP3, the major transcription factor associated with T_{reg} identity. Inside each memory and naïve population, the CD31 subsets were gated as shown in Figure 5.

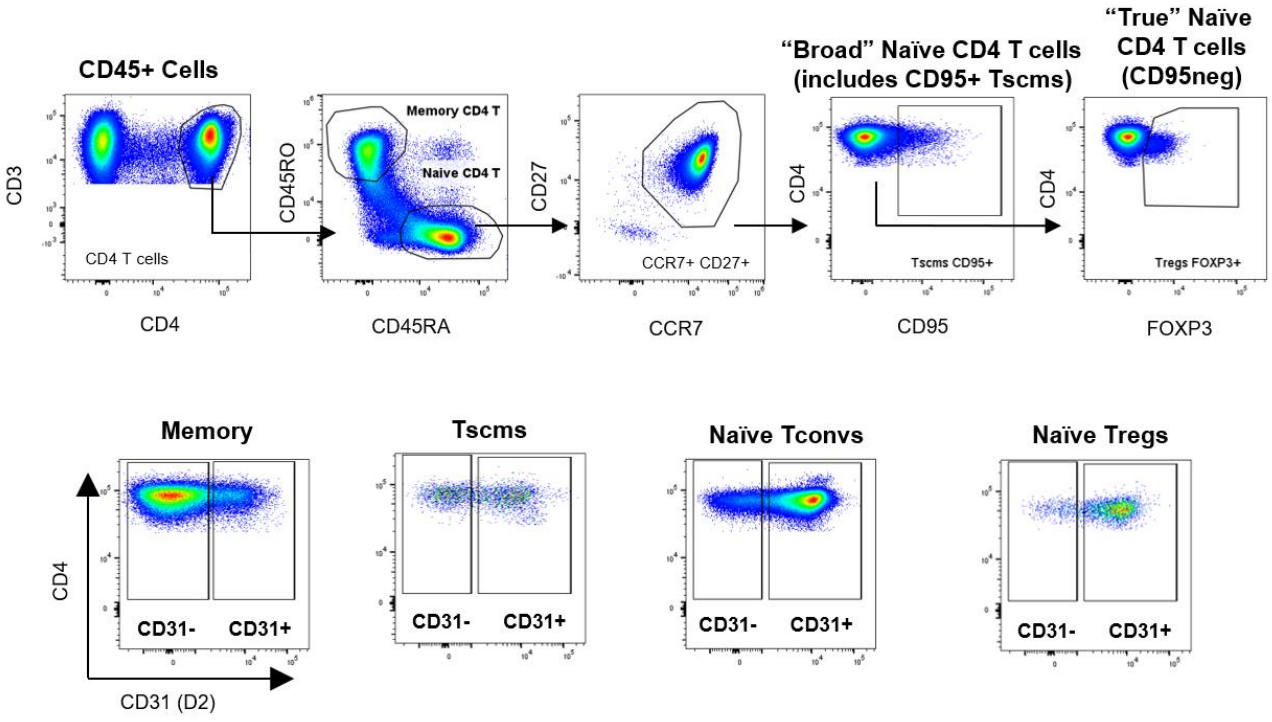


FIGURE 5: REPRESENTATIVE GATING STRATEGY TO IDENTIFY MAIN NAÏVE CD4 T CELL SUBPOPULATIONS AND CD31-DEFINED SUBSETS IN THE PAIRED SAMPLES OF BLOOD AND TONSIL ANALYSIS. (TOP) Gating strategy to identify naïve CD4 T cells (CD45RA+CD45RO-CCR7+CD27+) and further subpopulations. T_{scms} were identified based on CD95 expression and, on "true" naïve CD4 T cells, T_{convs} and T_{regs} were gated based on the expression of FOXP3. (DOWN) Expression of CD31 and gating strategy to identify CD31-defined subsets, across CD4 T cells memory and naïve subpopulations.

3.1 Characterization of CD31 expression in naïve and memory subsets.

Firstly, we assessed the expression of CD31 in both naïve and memory CD4 T cell populations (Figure 6). As expected, "true" naïve T_{convs} and T_{regs} have a high percentage of CD31-expressing cells, while the memory CD4 T cells show the lowest percentage of CD31+ cells (Figure 6.A). However, the percentage of CD31+ within the T_{scms} is different from both the naïve and the memory populations (Figure 6.A). No significant differences were found when comparing the percentages between Blood and Tonsil within the 4 populations (Figure 6.A).

Next, we focused on the expression levels of CD31, in the gated CD31-expressing cells of each population, which, as discussed before, is correlated with differential levels of basal expression of NF-kB and, consequently, IL-7 homeostatic response[2]. We found a significantly higher MFI (Median Fluorescence Intensity) in the tonsils, compared with their paired blood samples, in both T_{scms} and Naïve T_{convs}, while within T_{regs}, although the trend was similar, it failed to reach statistically significant thresholds (Figure 6.B). In the tonsil samples only, we also observed that T_{scms} have a higher MFI than memory CD31+ cells, although not significantly different from Naïve T_{convs} and T_{regs} (Figure 6.B). Tonsillar Naïve T_{convs} CD31+ cells also show a higher MFI when compared with Naïve T_{regs} (Figure 6.B).

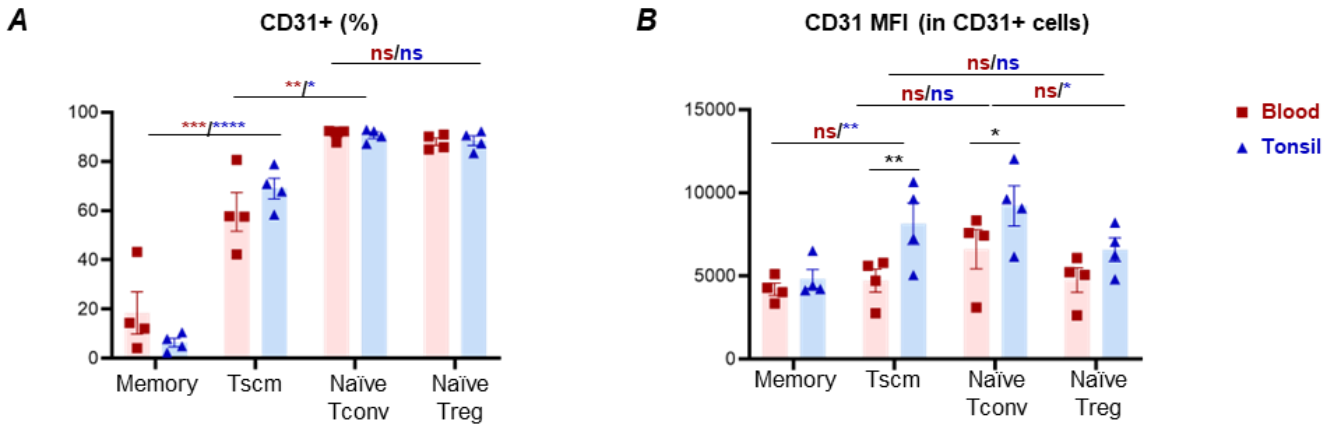


FIGURE 6: CD31 EXPRESSION IN CD4 MEMORY AND NAÏVE SUBPOPULATIONS IN BLOOD AND TONSIL. (A) Percentage (%) of CD31 expressing cells with the subpopulations of the memory and naïve CD4 T cell compartment. (B) CD31 MFI within CD31-expressing cells across the CD4 memory and naïve subpopulations. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on paired samples with two-way ANOVA and Sidak's multiple comparison (Blood vs Tonsil) or Tukey's multiple comparisons tests (to compare between CD4 T cell populations). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001).

3.2 Assessing phenotypical differences between CD31 subsets within naïve CD4 T_{convs}: T cell and naïve related markers.

To further characterize the CD31 subsets within the "true" naïve CD4 T cell compartment, we compared the expression levels of different T cell and naïve T cell-related markers in the CD31-defined subsets within naïve CD4 T_{convs} (Figure 7). We found that the CD31-expressing T cells mildly displayed a higher MFI of T cell

and naïve T cell-related, including CD3, CCR7, CD27 and CD38. When comparing the expression of the same markers between sample types, most of them, including CD45RA, CCR7 and CD5, have a higher expression in the blood samples.

When evaluating the frequency of cells expressing homing/activation markers, we found, as expected, a higher percentage of CD69 expressing cells in the tonsillar naïve T_{convs}, reflecting its function within tissues, with the percentage being marginally higher in the $CD31^{neg}$ subset[94]. However, the percentage of CXCR3+ cells is consistent across samples, being also higher on the $CD31^{neg}$ subset of naïve CD4 T cells.

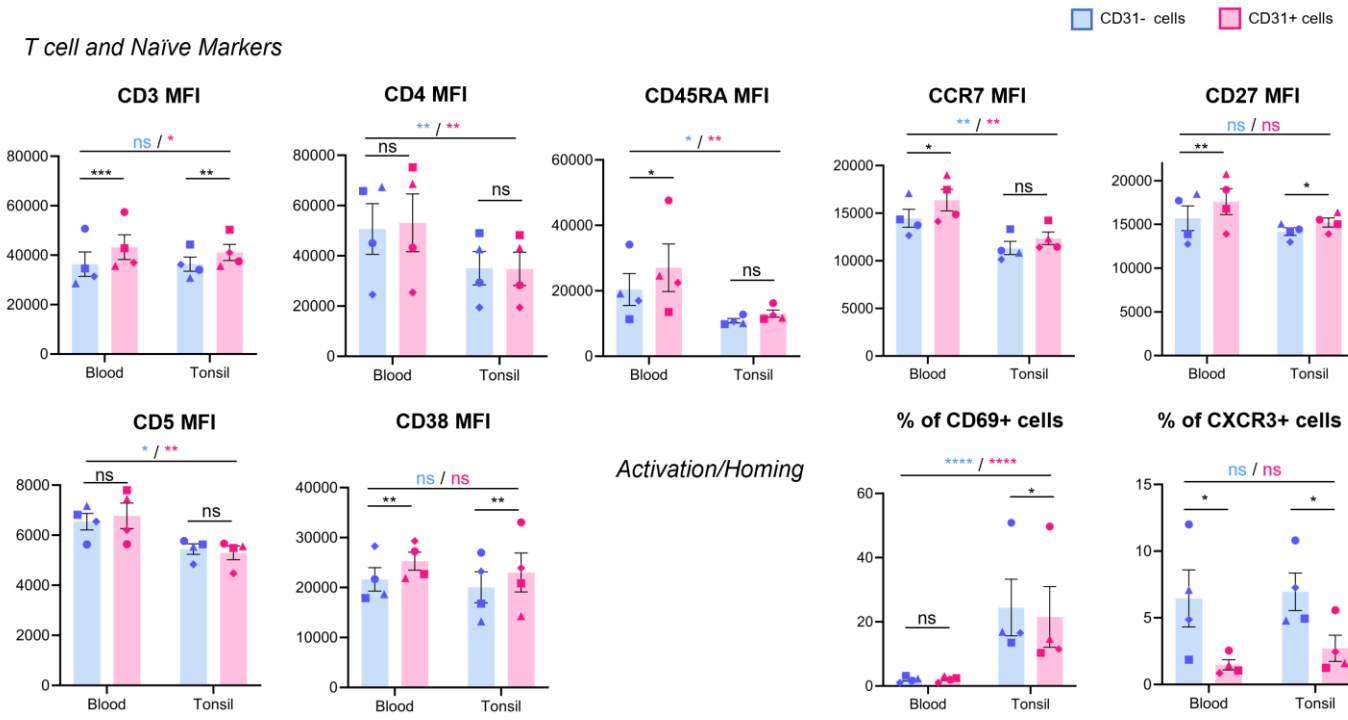


FIGURE 7: EXPRESSION OF T CELL AND NAÏVE CD4 T CELL-RELATED MARKERS IN NAÏVE CD4 T_{CONVS} CD31-DEFINED SUBSETS, ACROSS BLOOD AND TONSIL. MFI of the markers displayed (CD3, CD4, CD45RA, CCR7, CD27, CD5 and CD38) and the percentage of CD69 and CXCX3 (see supplemental figure 1 for gating strategy) expressing cells measured in the CD31 subsets of naïve CD4 Tconvs, in the paired samples of blood and tonsil. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on paired samples with two-way ANOVA and Sidak's multiple comparison tests (Blood vs Tonsil and CD31neg vs CD31+ cells). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001).

3.3 Assessing differences between CD31 subsets within naïve CD4 T_{convs}: IL-7 and Wnt signalling related markers.

We also investigated the expression of markers related to IL-7 and Wnt signalling pathways on CD31 subsets of naïve CD4 T_{convs} (Figure 8). As described in previous studies from our group, we observed a higher expression of CD127, the IL-7 receptor, in CD31+ cells[30]. This difference is better observed in the blood samples, where the CD127 MFI is higher than in the tonsils (Figure 8.A). Nevertheless, the observed differences in the expression of CD127 do not extrapolate to the expression levels of Bcl-2. Indeed, the measured MFI of Bcl-2 does not reveal significant differences across CD31 subsets or sample types (Figure 8.A). As expected,

only a small fraction of Ki-67-expressing cells were observed. The highest percentage of proliferating cells was found on tonsillar CD31+ T_{convs}, reaching a statistical significance when compared to peripherical blood CD31+ cells (Figure 8.A).

Focusing on the selected Wnt signalling-related markers, we found that the percentage of detectable β-catenin expressing cells is very low and does not show any significant difference when comparing CD31 subsets or sample types (Figure 8.B)[55,56]. The measured MFI of TCF1, however, is marginally lower in the blood naïve CD4 T cells and, when comparing between peripherical CD31 subsets, it reveals a small, even if statistically significant, difference (Figure 8.B).

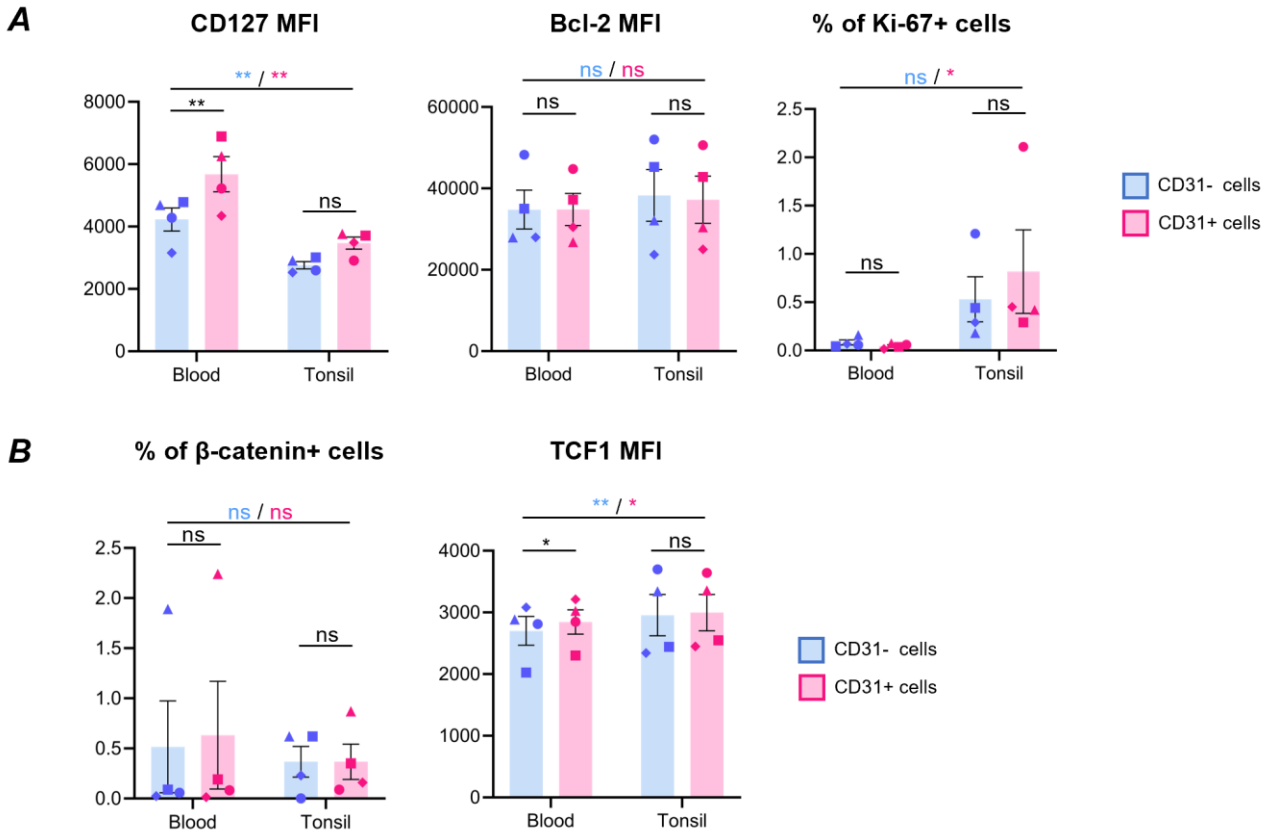


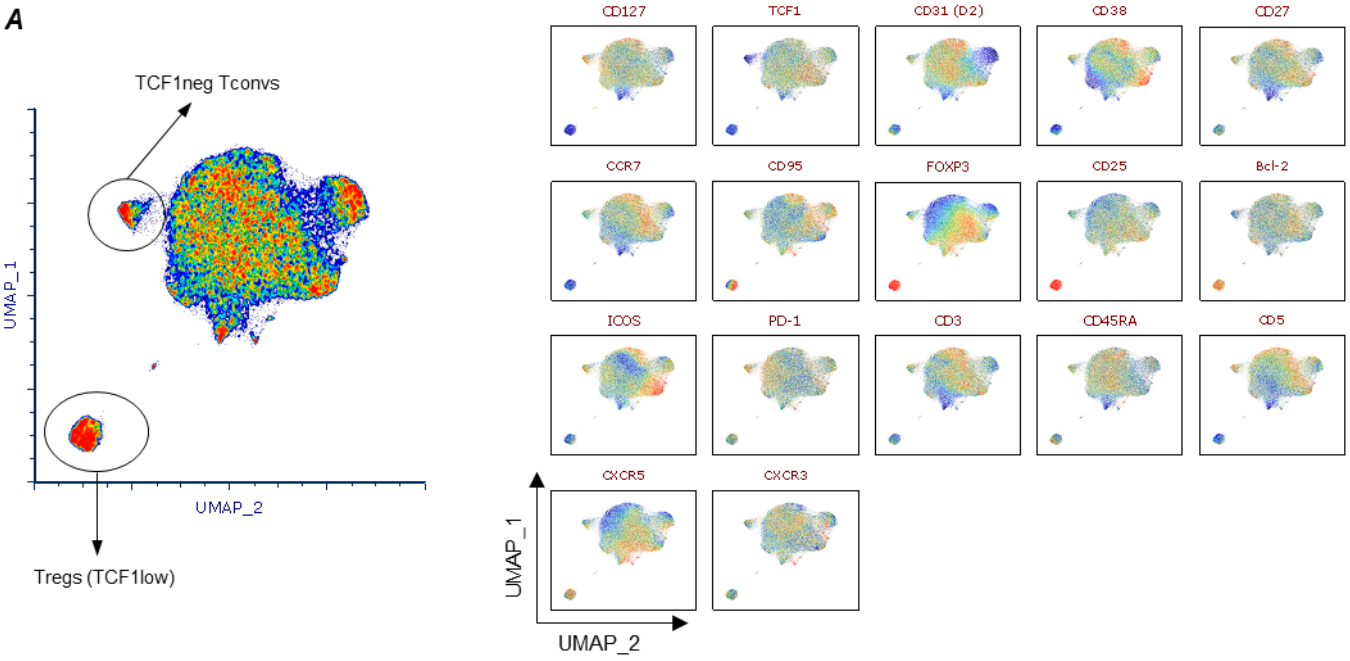
FIGURE 8: EXPRESSION OF IL-7 AND WNT SIGNALLING-RELATED MARKERS WITHIN NAÏVE CD4 T_{CONVS} CD31-DEFINED SUBSETS, ACROSS BLOOD AND TONSIL. (A) Measured MFI of CD127 and Bcl-2, and percentage of Ki-67+ proliferating cells in the gated subsets defined by CD31 expression within naïve CD4 Tconvs. (B) Percentage of β-catenin expressing cells and measured MFI of TCF1 expression in the CD31-defined subsets of naïve CD4 T cells (see supplemental figure 1 for the gating strategy of Ki-67 and β-catenin expressing cells). Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on paired samples with two-way ANOVA and Sidak's multiple comparison tests (Blood vs Tonsil and CD31neg vs CD31+ cells). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001).

3.4 Characterization of total naïve CD4 T cell heterogeneity using Uniform Manifold Approximation and Projection (UMAP) analysis.

To take full advantage of our comprehensive antibody panel (Table 1) we performed an unsupervised analysis, in parallel to the analysis shown above, in an attempt to better characterize the heterogeneity within

the naïve CD4 T cell compartment (Figure 9). For this purpose, the gated populations of "Broad" Naïve CD4 T cells from the 4 subjects were concatenated with the same number of events from each one (Supplemental Figure 2.A). Given the different sample origins, the tonsillar and peripherical populations were analysed separately, with one of the blood samples being excluded due to issues related to the required axis scaling of the marker's expression for the unsupervised analysis. Samples from the different donors display a similar population distribution and the identified subpopulations are present in each of the individuals analysed (Supplemental Figure 3). In both UMAPs, naïve T_{convs} and T_{regs} are clearly segregated (Figure 9.A and 9.B). The CD31 subsets, although identifiable in both UMAPs, do not seem to be correlated with any other marker's expression (Figure 9.A and 9.B).

When comparing both UMAPs, we observed that tonsillar naïve CD4 T cells show more distinct subpopulations. Furthermore, we found that some of these subpopulations could be specially defined by different levels of TCF1 expression (Figure 9.B). We found that T_{regs} could be additionally characterized by low expression levels of TCF1 (Figure 9.A and 9.B). On T_{convs}, we were able to define to identify a small subpopulation of CD31+ T_{convs} that does not appear to express TCF1, in both sample types, while, exclusively in tonsils, we found a subpopulation of CD69-expressing T_{convs} associated with a high-level expression of TCF1 (Figure 9.A and 9.B).



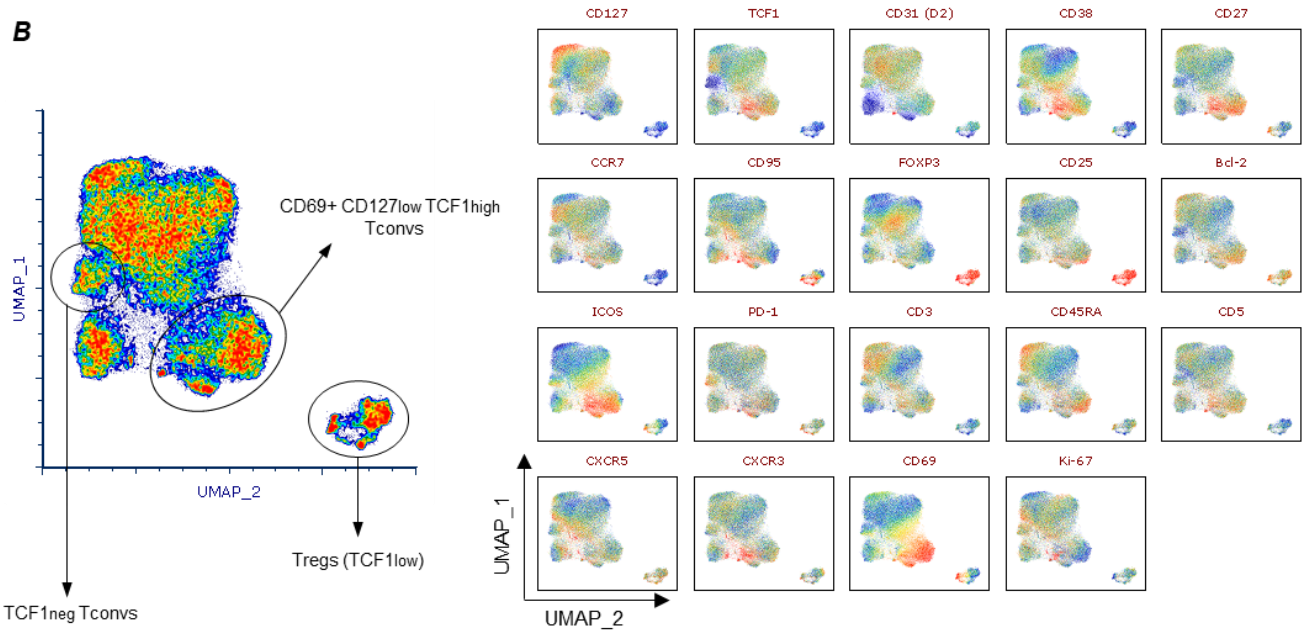


FIGURE 9: HETEROGENEITY WITHIN THE NAÏVE CD4 T CELL COMPARTMENT, UNSUPERVISED APPROACH. (A) Blood and (B) Tonsil "Broad" naïve CD4 T cell compartments' heterogeneity represented in a Uniform Manifold Approximation and Projection (UMAP) with annotations for the subsets with differential expression of TCF1. On the right, different plots shown are projections of the noted marker in the overall UMAP.

To better describe the TCF1-defined subsets in the tonsils, we gated them out of the Tonsil UMAP and created a heatmap, shown in Figure 10.A, allowing us to display the relative expression of other related markers. Additionally, we search to confirm our main observations through conventional analysis (Figure 10, see Supplemental Figure 2 for gating strategy). We show that, indeed, T_{regs} have a lower expression of TCF1 than T_{convs}, further confirmed by the measured MFI (Figure 10.A and 10.B). We also found that the TCF1 negative T_{convs} displayed slightly lower levels of Bcl-2, although demonstrating normal expression levels of CD127 (Figure 10.A and 10.C).

Importantly, we also observed that the CD4 naïve T_{convs} with high expression of CD69 and TCF1 showed other distinct phenotypical differences. We found that this population has a high expression of CD38 and ICOS but a lower expression of CD127, suggesting ongoing response to IL-7. However, we could not detect a significantly different expression of Bcl-2 (Figure 10.D). Even though this subset includes CD31+ and CD31^{neg} naïve T_{convs}, CD31 expression does not correlate with the expression of the characteristic markers of this subset (Supplemental Figure 4).

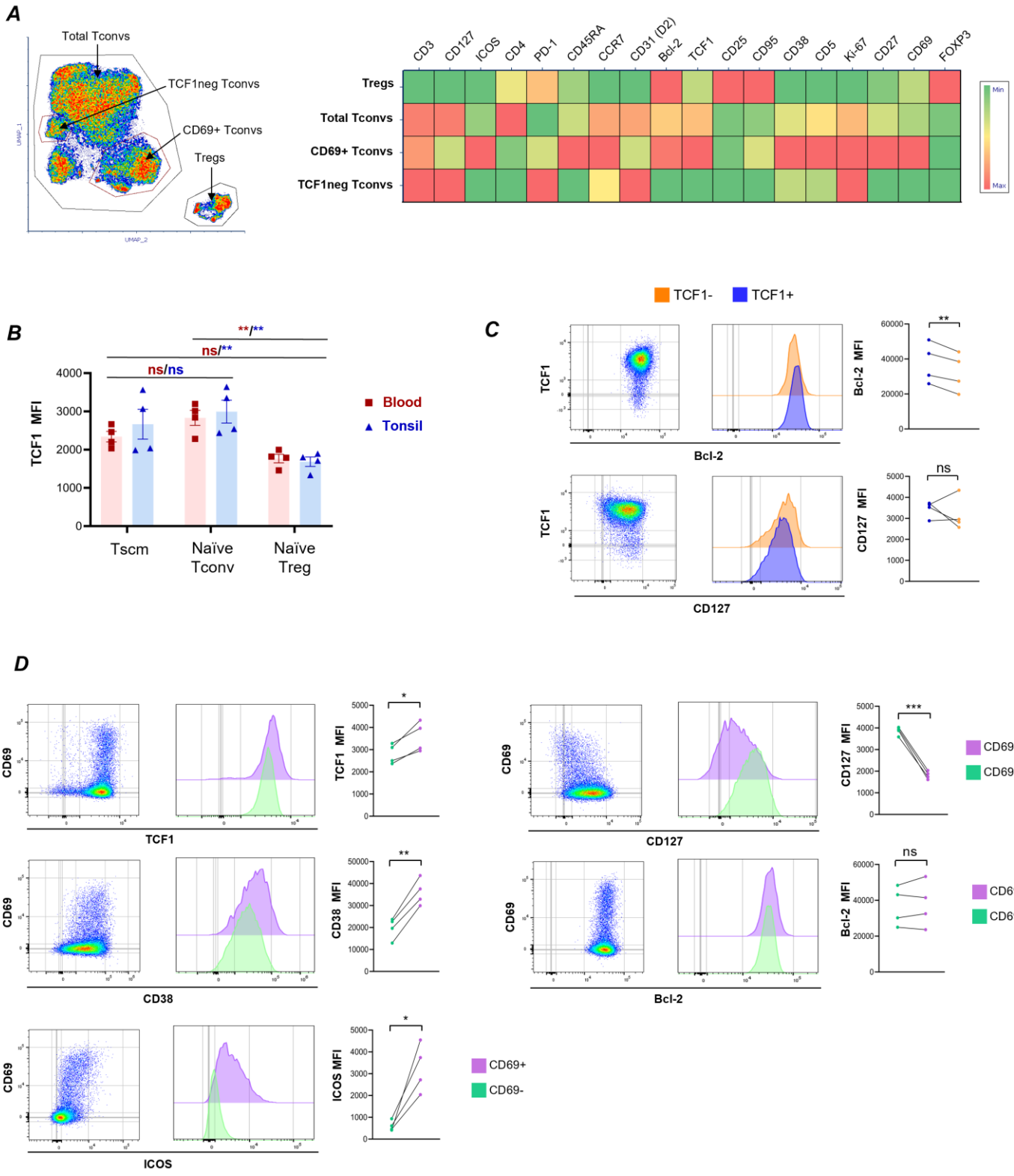


FIGURE 10: CHARACTERIZATION OF TCF1-DEFINED SUBPOPULATIONS PHENOTYPE IN THE TONSILLAR BROAD NAÏVE CD4 T CELL COMPARTMENT. (A) Tonsillar Naïve CD4 T cells' UMAP (left) with gated populations of the main TCF1-defined subpopulations and heatmap (right) displaying the relative expression of the markers in the corresponding subpopulations. (B) Measured MFI of TCF1 within the populations of Tscms, naïve Tconvs e naïve Tregs in both blood and tonsil samples (two-way ANOVA and Tukey's multiple comparison tests). (C) Representative dot-plots and histograms of the expression of Bcl-2 and CD127 and measured MFI (paired T test) of the respective markers in the TCF1 negative and positive subpopulations of naïve CD4 Tconvs (see supplemental figure 2.B for gating strategy to identify TCF1 negative cells). (D) Representative dot-plots and histograms of the expression of TCF1, CD38, ICOS, CD127 and Bcl-2 and measured MFI (paired T test) of the respective markers in the CD69 negative and positive subpopulations of naïve CD4 Tconvs

(see supplemental figure 2.B for gating strategy to identify CD69-expressing cells). Individual samples are depicted. The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001).

4. Optimization of CD31 and Wnt Signalling Modulators.

Given our results and the suggested relevance of Wnt/TCF1 in naïve CD4 T cells, we searched to investigate the crosstalk between IL-7 signalling, CD31 and Wnt/TCF1, using *in vitro* modulation of both CD31 and Wnt signalling in sorted naïve CD4 Tconvs[55]. We decided to start with a one-trial experiment so that we could find operational doses of modulators for our experiments, based on initial dosages from the literature[90,91,95–97]. The gating strategy applied to identify naïve CD4 T_{convs}, as well, the CD40L and CD69 expressing cells upon TCR activation, was done as shown in Figure 11.

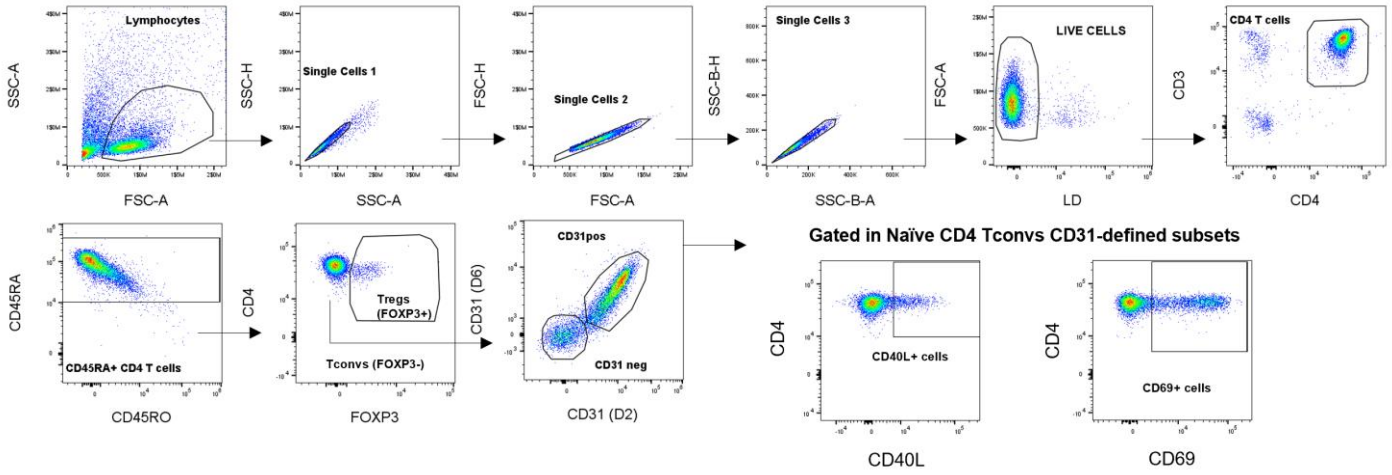


FIGURE 11: REPRESENTATIVE GATING STRATEGY USED IN THE OPTIMIZATION OF CD31 AND WNT SIGNALLING MODULATION. After culture, the naïve CD4 Tconvs were identified based solely on the expression of CD4 and CD45RA. In the following point 4.1, the quantification of activated cells, after TCR stimulation, was based on CD40L and CD69 expression, as shown here.

4.1 CD31 Modulators.

Our main goal when modulating CD31 signalling was to verify if it would alter the short-time response to TCR stimulation. We opted to use 3 different modulators. To promote CD31 signalling, we selected the recombinant human CD31 peptide (CD31 agonist), which was reported to engage in homophilic binding and trigger CD31 signalling in cells[90,98]. On the other hand, to inhibit CD31 signalling, we used an anti-CD31 antibody (CD31 Blocking Ab), reported to antagonize CD31 dependent signalling and, in order to attempt pharmacological inhibition of CD31-dependent signals, we have chosen to use SHP099, which inhibits SHP-2, present in the downstream pathway of CD31 signalling[82,95]. We evaluated the effects in this modulation with flow cytometry readouts of activation, namely CD40L and CD69 upregulation (see Table 2 for Antibody Panel).

After a 3 hours culture, both the CD31 peptide agonist and the blocking antibody do not show clear results, as the induced changes seem to affect both populations similarly (Figure 12.A). Regarding the CD31 agonist

peptide, we opted to choose the concentration where we observed the biggest difference between the CD31 defined subsets. In the modulation by the anti-CD31 blocking antibody, we found that our chosen concentrations were lacking, because, although we observed a tendency for a decrease in CD40L expression with increasing doses within CD31+ cells, CD40L expression did not reach similar numbers to those observed in CD31 negative cells. We opted to increase the concentration in subsequent experiments. However, we found that SHP099 is able to abrogate T cell activation in the CD31 expressing subset to similar numbers observed in the CD31^{neg} counterpart. Taking this data and published results, we decided to use these modulators in subsequent experiments with the following concentrations: 25 µg/mL for CD31 agonist peptide, 50 µM for SHP099 and 50 µg/mL for the anti-CD31 blocking Ab.

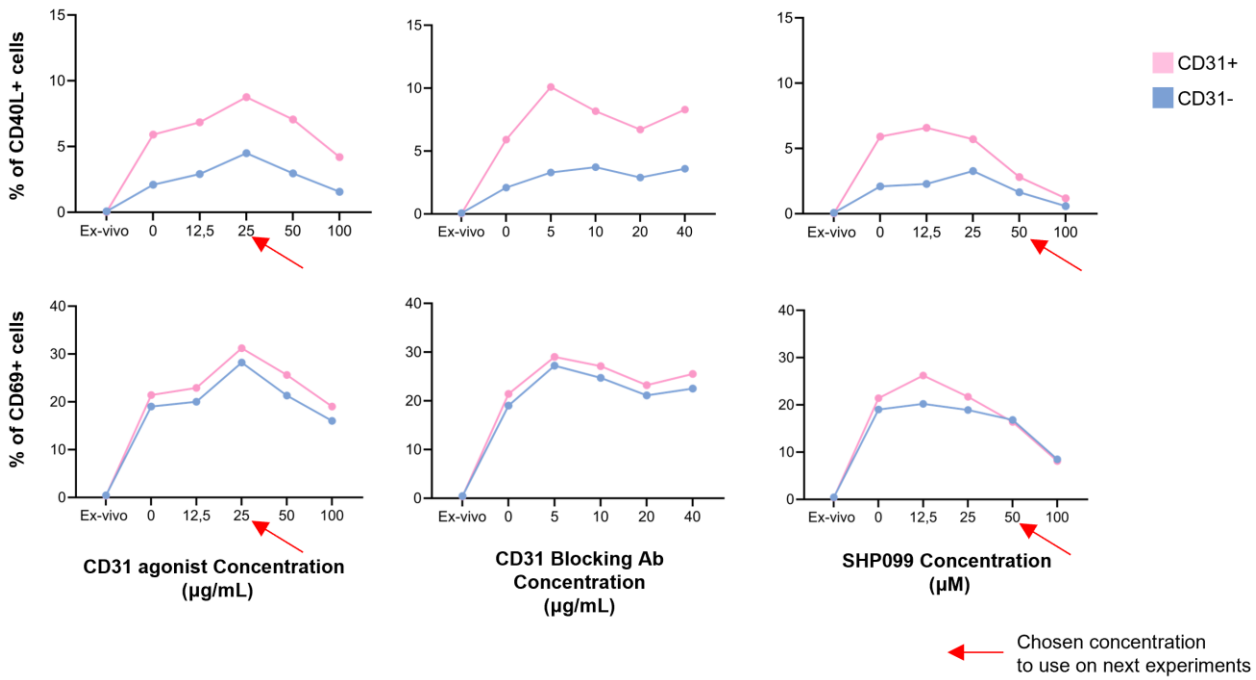


FIGURE 12: QUANTIFICATION OF ACTIVATED CELLS UPON CD31 MODULATION (OPTIMIZATION). Percentage of (A) CD40L and (B) CD69 expressing cells, within the CD31-defined naïve CD4 Tconv subsets, after a 3 hour culture with TCR stimulation (at a 1:8 beads to cells ratio) and respective modulation on CD31 signalling.

4.2 Wnt Modulators.

To better understand Wnt signalling's possible crosstalk with the response to homeostatic stimuli and its role in establishing the heterogeneity within the naïve CD4 T_{conv} population, we search to modulate this signalling pathway in homeostatic stimulation signal stimulation experiments. Similarly to the optimization experiment done for the CD31 modulators, we opted to test increasing concentrations of DKK-1, a physiological inhibitor of Wnt signalling, and CHIR99021, which blocks the inhibitory function of GSK3, leading to Wnt activation (Figure 13), analysing the effects after 3 days of culture using flow cytometry-based readouts (see Table 3 for Antibody Panel). Although our main focus is response to IL-7 in absence of TCR stimulation, we also evaluated impacts in presence of TCR stimulation.

We were able to establish the optimal concentrations for both modulators for IL-7 stimulation based on β-catenin and TCF1 MFIs (Figure 13). After the 3 days culture, even though it does not clearly alter TCF1 expression, DKK-1 modulation decreases β-catenin MFI (Figure 13.A). On the other side, Wnt activation with the GSK3 inhibitor shows both an increase of TCF1 and β-catenin expression, consistent with the increasing concentration of the modulator (Figure 13.B). Based on these results we decided to continue our further experiments with the following concentrations: 50 µM for DKK-1 and 3µM for GSK3 inhibitor.

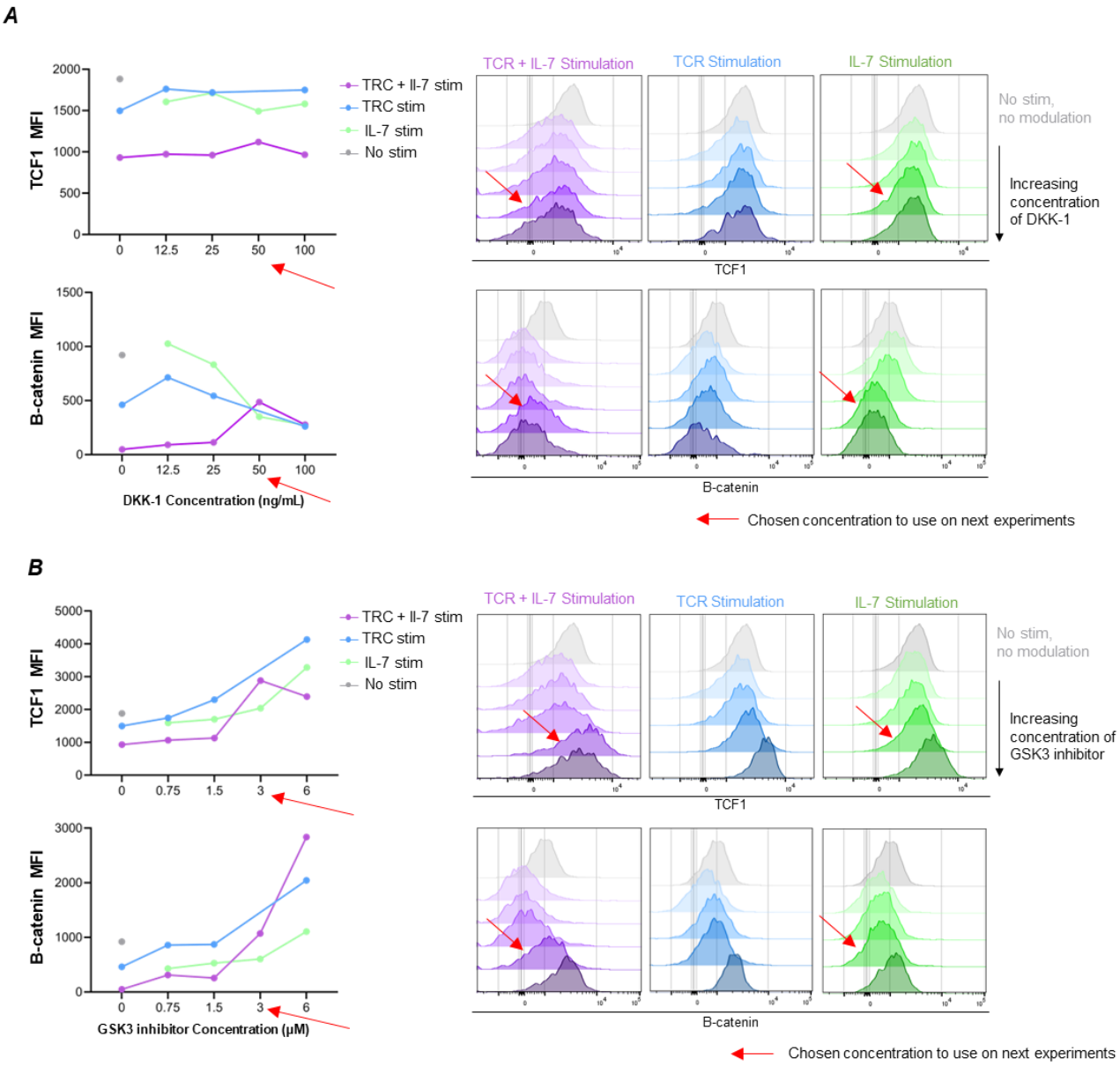


FIGURE 13: MODULATION OF THE WNT SIGNALLING PATHWAY (OPTIMIZATION). Measured MFI and respective histograms of TCF1 and β-catenin, after a 3-day culture with TCR stimulation (at a 1:32 beads to cells ratio) and/or IL-7 stimulation (10ng/ml), and modulation by (A) DKK-1 and (B) GSK3 inhibitor, CHIR99021.

5. Evaluating short-time alterations in TCR activation of naïve CD4 T_{convs} upon CD31 modulation.

To evaluate the role of CD31 signalling in the early response to homeostatic signals, magnetically sorted untouched naïve CD4 T_{convs} were cultured in the presence of the CD31 Modulators, CD31 agonist peptide, CD31 Blocking antibody and SHP099 for 3 Hours upon TCR and/or IL-7 stimulation (Figure 14.A). The CD4 naïve T_{convs} CD31 subsets and activated cells were identified as shown in Figure 14.B and Supplemental Figure 5 (see Table 4 for antibody panel).

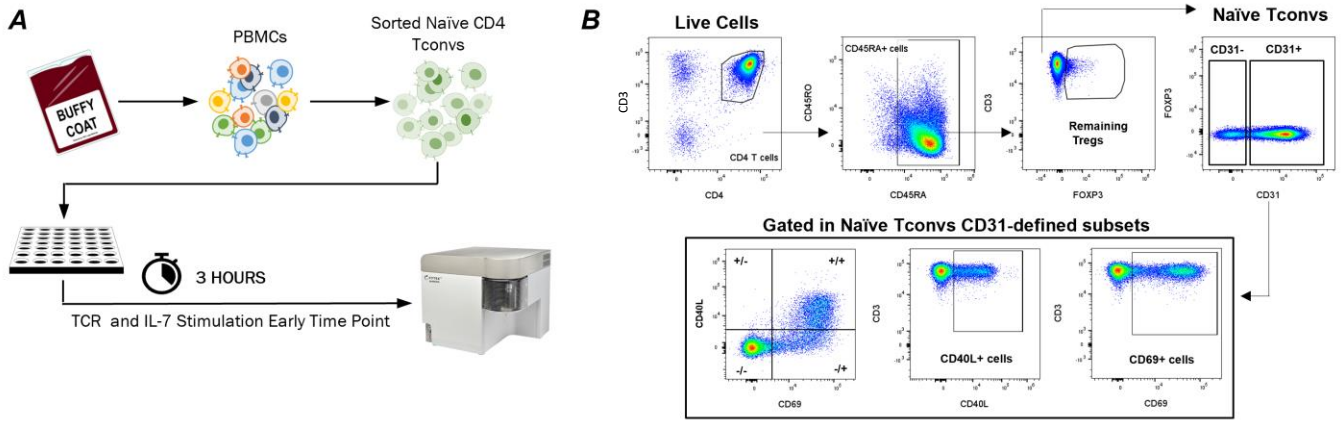


FIGURE 14. WORKFLOW TO DETERMINE TCR ACTIVATION CHANGES UPON CD31 MODULATION. (A)Illustration of the workflow preformed to study the impact of CD31 signalling on short-term response to homeostatic signalling of TCR and IL-7. Magnetically sorted naïve CD4 Tconvs, collected from buffy coats, were cultured for 3 hours with one of the CD31 modulators (CD31 agonist peptide, SHP099 and CD31 Blocking Ab) in the presence of TCR and/or IL-7. Afterwards, the cells were stained (see Table 4) and acquired via flow cytometry (B) Gating strategy used to identify the CD31-defined naïve CD4 T conv subsets and quantify the activated cells upon TCR stimulation, based on the individual or combined expression of CD69 and CD40L expression.

5.1. CD31 expression changes after CD31 modulation and TCR activation.

To confirm changes in CD31 expression after the 3 hours culture with CD31 modulation, we analysed both the percentage of CD31 expressing cells and the CD31 MFI in that same population. We did not find any alterations in the CD31 expression, but the modulation with CD31 agonist interferes with the recognition of the domain 2 (D2) of CD31 by the antibody (Figure 15.A). This caveat is shown to increase with higher concentrations of the CD31 agonist (Supplemental Figure 6). Curiously, however, the recognition is partially recovered in presence of TCR stimulation (Figure 15.B).

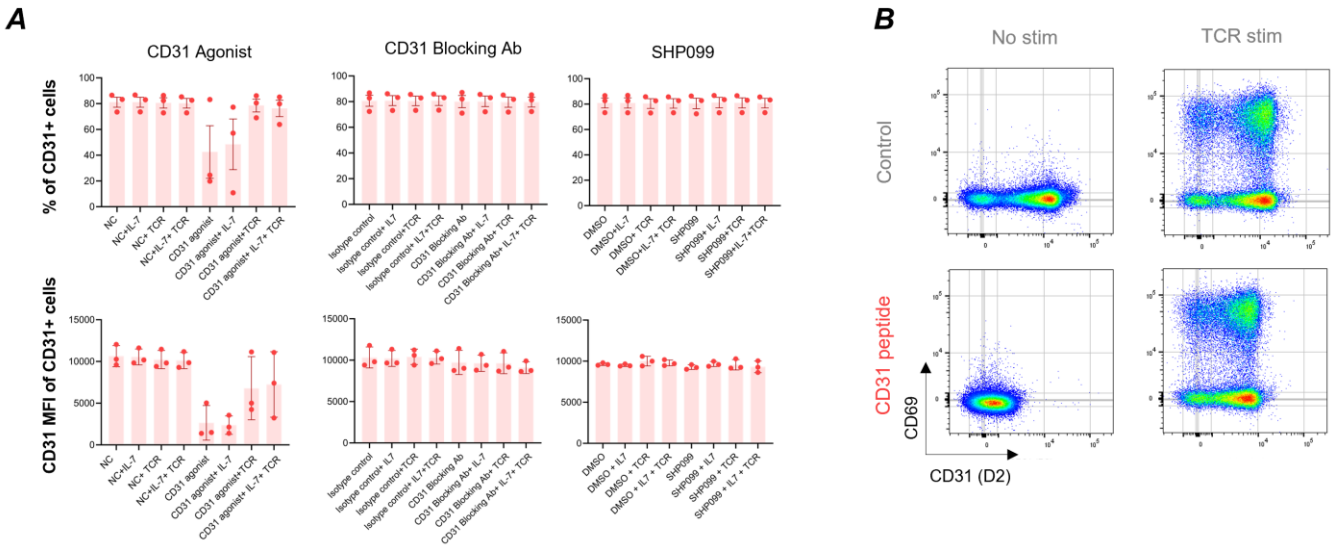


FIGURE 15: CD31 EXPRESSION UPON TCR STIMULATION AND CD31 MODULATION. (A, Top) Percentage of CD31 expressing cells and (A, down) CD31 MFI within this subset after a 3 hour culture with TCR and/or IL-7 stimulation and CD31 signalling Modulation. (B) Representative dot-plots showcasing the antibody recognition (see Table 4 for antibody reference) of domain 2 of CD31 upon modulation with the agonist CD31 peptide, with and without TCR stimulation. NC: negative control; CD31 agonist: recombinant human CD31 peptide; CD31 blocking Ab: anti-CD31 blocking antibody; SHP099: SHP-2 selective inhibitor; TCR: TCR-stimulation with CD3/CD28 beads.

5.2. Identifying alterations in activation markers' expression upon CD31 modulation.

When assessing the T cell activation changes upon CD31 modulation, we observed that the CD31 agonist and blocking Ab did not alter the percentage of activated cells and the same occurred with SHP099. To further investigate alterations in T cell activation, we gated the naïve CD4 T_{convs} into quadrants based on the expression of both markers, CD69 and CD40L, and also measured the MFI of the markers (Figure 14, 16.B and 16.C). We observed that the modulation by SHP099 maybe be reflected in a decrease of the double positive cells (CD40L+CD69+), although the difference did not reach statistical significance (Figure 16.B). When measuring the expression levels of the activated cells, we observed that both CD69 and CD40L MFIs are also substantially lower when modulated by SHP099, even though it was only statistical different for comparisons on CD69 MFI (Figure 16.C). The effects described were not CD31-specific, since they occurred for both CD31 naïve subsets and were also independent of the presence or absence of IL-7 (Figure 16.B and 16.C). Concerning these same parameters, we could not observe an impact of either CD31 agonist or blocker in naïve CD4 T cell activation.

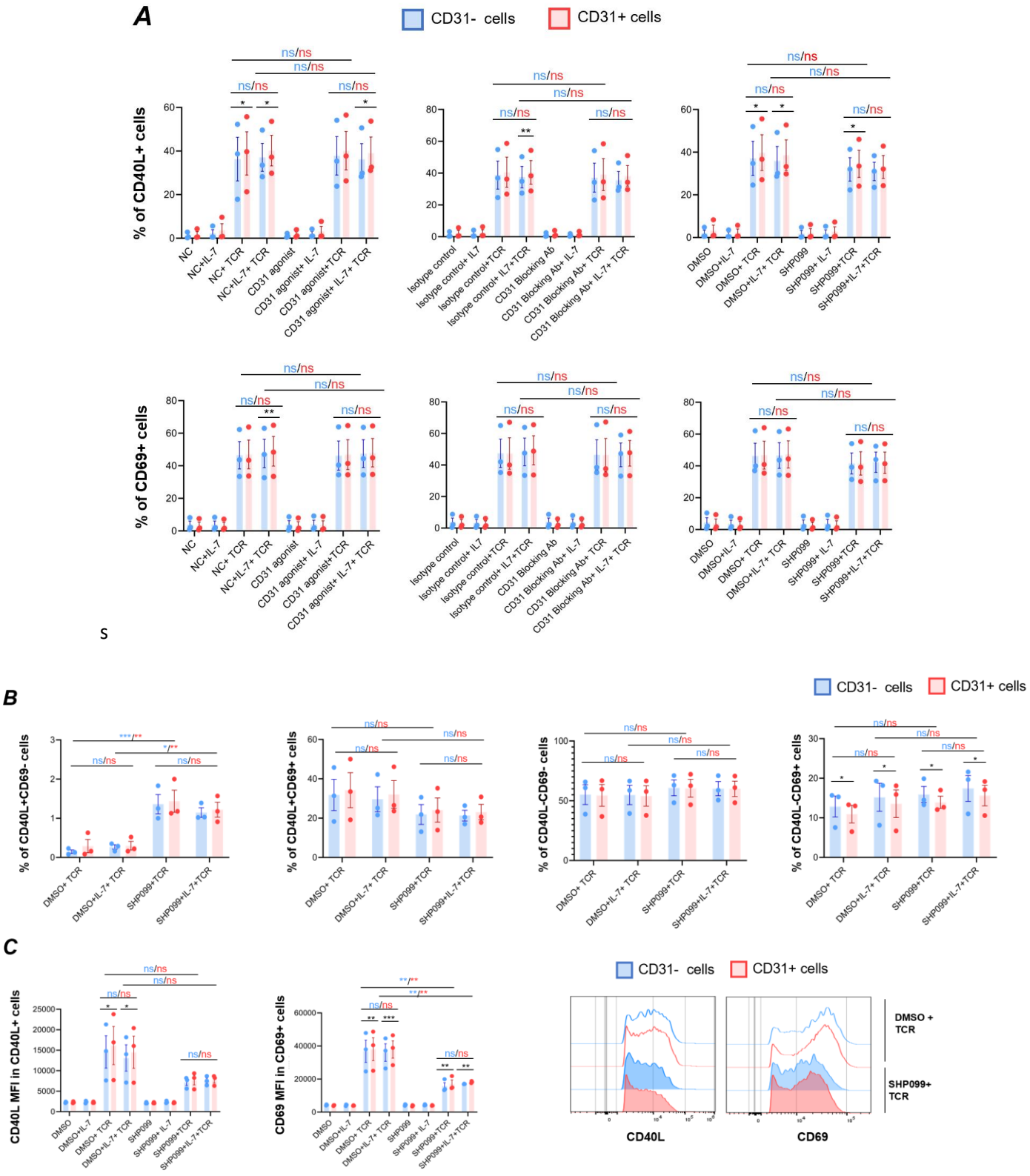


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FIGURE 16: TCR ACTIVATION IS ALTERED BY SHP099 MODULATION. (A) Quantification of CD40L and CD69-expressing activated cells with CD31-defined subsets of naïve CD4 Tconvs, after 3 hours culture with CD31 modulation (CD31 agonist peptide, anti-CD31 Blocking Ab and SHP099) and TCR/IL-7 stimulation. (B) Quantification of the activation subsets, based on the combined expression of CD69 and CD40L, in the CD31-defined subsets of naïve CD4 Tconvs, after 3 hours culture with SHP099 with TCR (and with or without IL-7) stimulation. (C, left) Measured MFI of CD40L and CD69 within the CD31-defined naïve CD4 Tconvs that express the respective markers upon the modulation by SHP099 with TCR and/or IL-7 stimulation and (right) representative dot-plots displaying CD40L and

CD69 expression, after 3 hours culture with SHP099 in the presence of TCR stimulation. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on matched samples with two-way ANOVA and Sidak's multiple comparison (CD31neg vs CD31+ cells) and Tukey's multiple comparison tests (compare culture conditions). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001).NC: negative control; CD31 agonist: recombinant human CD31 peptide; CD31 blocking Ab: anti-CD31 blocking antibody; SHP099: SHP-2 selective inhibitor; TCR: TCR-stimulation with CD3/CD28 beads.

5.3. Identifying alterations in IL-7 and Wnt signalling-related markers upon CD31 modulation.

When further assessing the effects of CD31 modulation, we also measured the expression levels of IL-7 and Wnt signalling-related markers. The CD31 agonist peptide and the anti-CD31 blocking antibody did not cause any significant alterations on these markers (Figure 17.A and Supplemental Figure 3). However, we found that SHP099 decreases CD127 expression of CD31+ naïve CD4 T_{convs}, in the presence of TCR stimulation only (statistically significant) and $CD31^{neg}$ (not reaching statistical significance) (Figure 17.A and 17.B). No additional effects were observed for Bcl-2 or TCF1 MFIs (Supplemental Figure 7). Overall, our results do not show critical impacts of the modulations tested in these aspects of early response of naïve CD4 T cells to TCR and IL-7 stimulation.

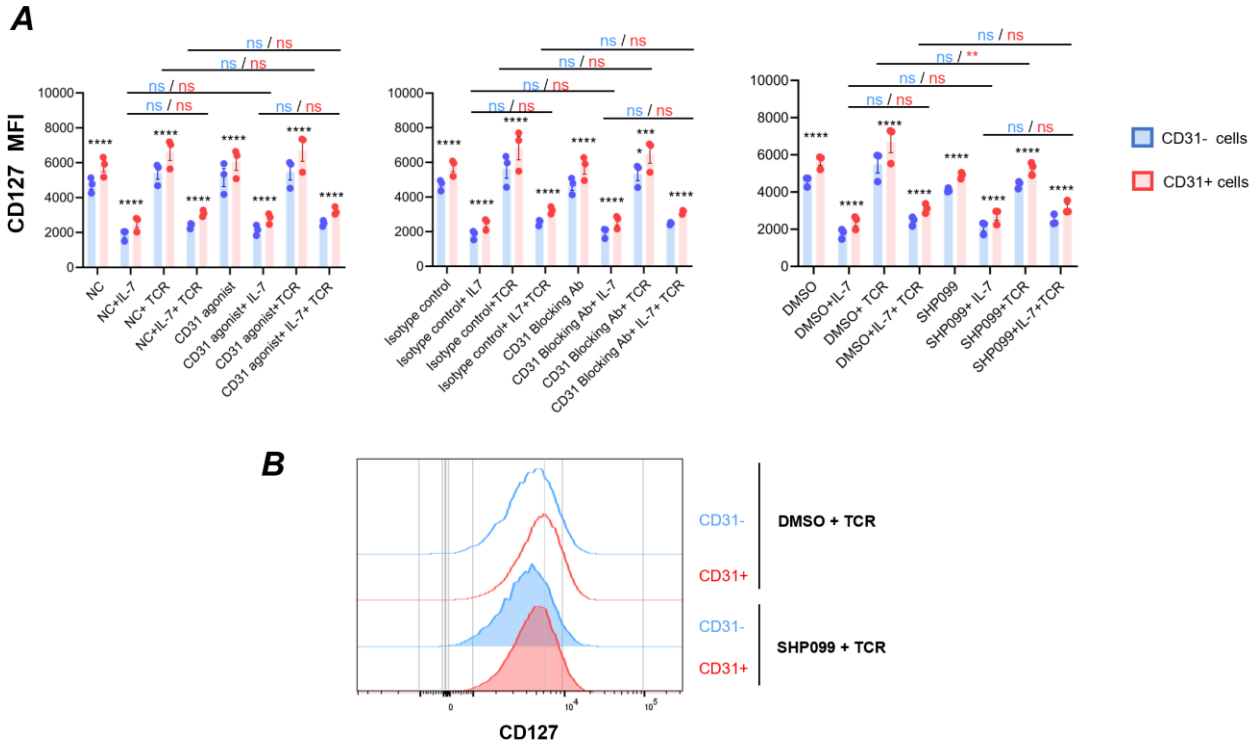


FIGURE 17: CD127 EXPRESSION IS ALTERED UPON TCR STIMULATION WHEN IN THE PRESENCE OF SHP099 MODULATION. (A) Measured MFI of CD127 within the CD31-defined naïve CD4 Tconvs, after the 3 hours culture with CD31 modulation in the presence of TCR and/or IL-7 stimulation. (B) Representative dot-plots displaying CD127 expression, after 3 hours culture with SHP099 in the presence of TCR stimulation. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on matched samples with two-way ANOVA and Sidak's multiple comparison (CD31neg vs CD31+ cells) and Tukey's multiple comparison tests (compare culture conditions). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001). NC: negative control; CD31 agonist: recombinant human CD31 peptide; CD31 blocking Ab: anti-CD31 blocking antibody; SHP099: SHP-2 selective inhibitor; TCR: TCR-stimulation with CD3/CD28 beads.

6. Wnt Modulation alters naïve CD4 T cell phenotype in response to IL-7 stimulation.

To assess the role of Wnt signalling in the homeostasis of naïve CD4 T cells, sorted naïve CD4 T_{convs} were cultured in the presence of the natural inhibitor of Wnt, DKK-1, or CHIR99021, a GSK3 inhibitor able to activate the canonical Wnt signalling. The modulators were used to confirm a possible role in both short-term (3 Hours) response to TCR and/or IL-7 stimulation and longer-term (7 Days) in IL-7 or IL-2 only stimulation. Readouts of stimulation and response were obtained by flow cytometry (Figure 18.A; for the short and long-term culture staining panels used, see Tables 5 and 6, respectively). It is important to note that IL-2 is also used in the long-term culture to establish a comparison with a cytokine that plays a minor role in absence of TCR stimulation, highlighting the IL-7-specific effects of the stimulation. To additionally confirm a possible differential response to the stimuli and modulation of the CD31-defined subsets, naïve CD4 T_{convs} were gated as shown in Figure 18.B and Supplemental Figure 5.

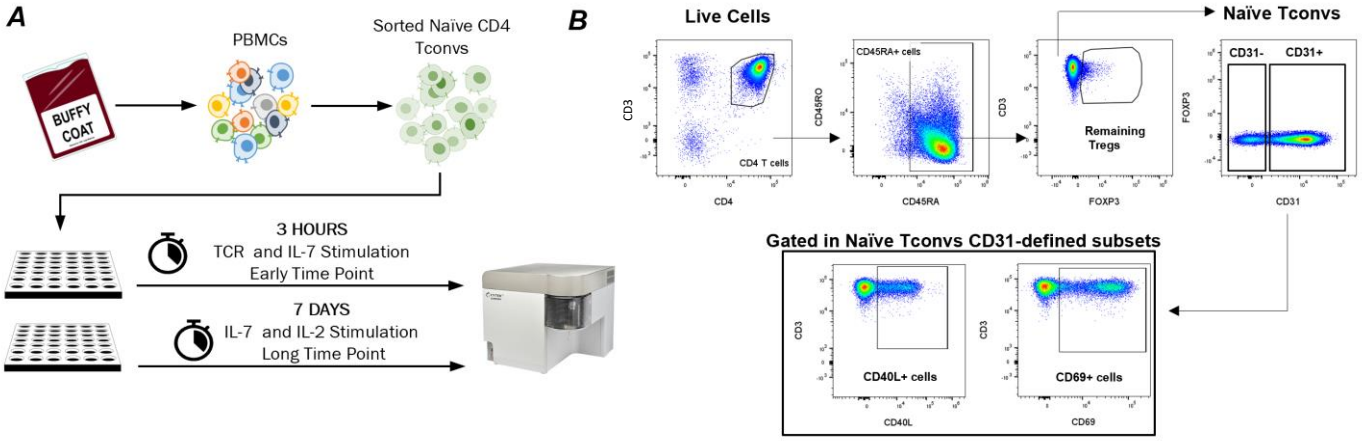


FIGURE 18: WNT MODULATION EXPERIMENT WORKFLOW. (A)Illustration of the workflow preformed to study the impact of CD31 signalling on short-term response to homeostatic signalling of TCR and IL-7. Magnetically sorted naïve CD4 Tconvs, collected from buffy coats, were cultured for 3 hours or 7 days with one of the Wnt modulators (GSK3 inhibitor and DKK-1). Afterwards, the cells were stained (see Table 4 and 5) and acquired via flow cytometry (B) Gating strategy used to identify the CD31-defined naïve CD4 T conv subsets and quantify the activated cells upon TCR stimulation, based on the individual expression of CD69 and CD40L expression.

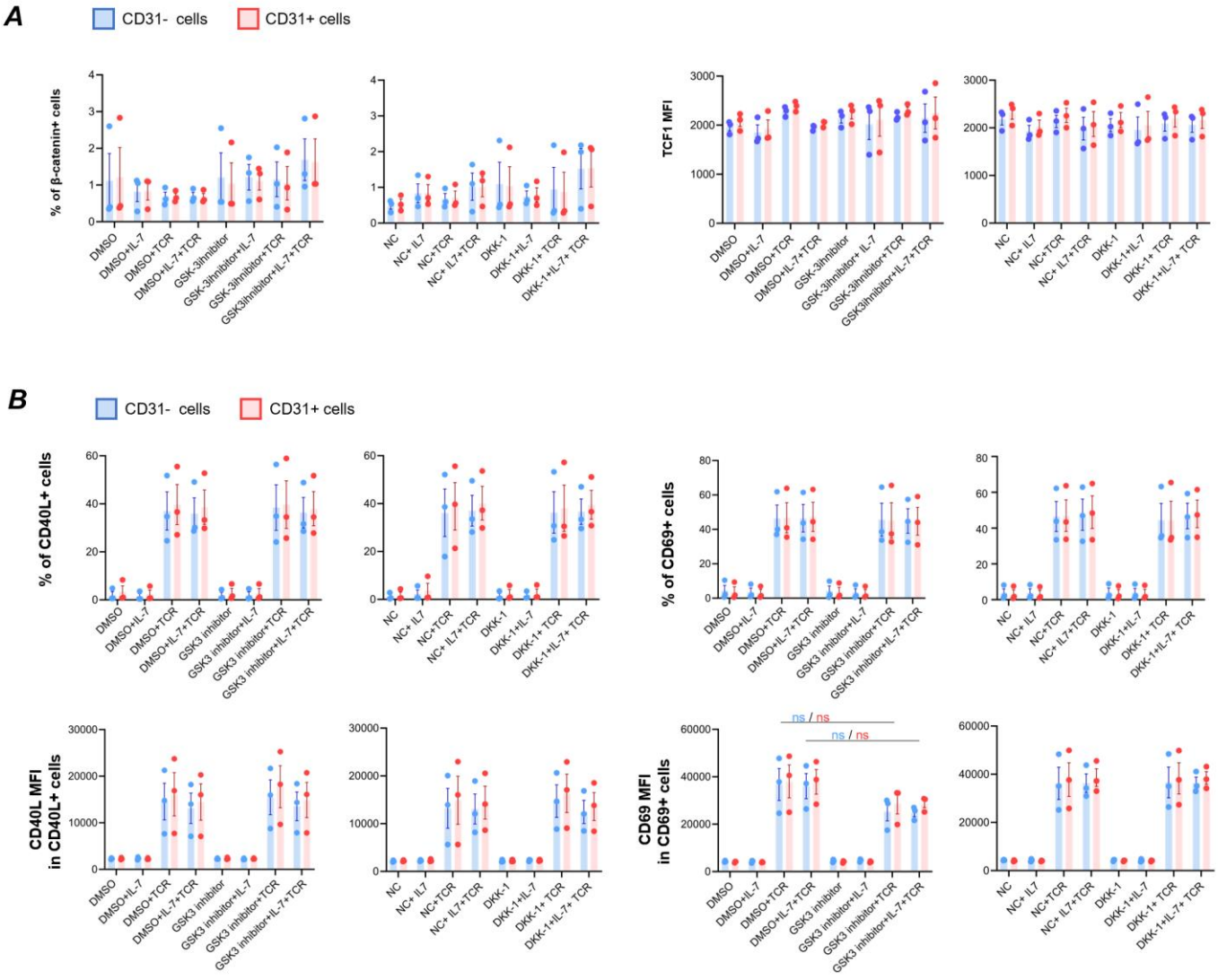
6.1. Early TCR and IL-7 response upon Wnt signalling modulation.

To confirm the modulation of the Wnt signalling upon adding the GSK3 inhibitor and DKK-1, we started by measuring the MFI of TCF1 and the percentage of β-catenin expressing cells. In the 3 hours time-point, we did not find evidence of Wnt modulation from both modulators (Figure 19.A). Moreover, when assessing CD31 expression, we did not find any alterations (Supplemental Figure 8.A).

We quantified then the percentages of activated cells, based on CD40L and CD69 expression (see Figure 18 for gating), to assess alterations in the short-term response to TCR stimulation. We did not find evidence that Wnt signalling modulation alters the activation of naïve T cells upon exposure to TCR stimulation (Figure 19.B). However, when we evaluated the expression level of these markers within activated cells, we found that CD69-

expressing activated cells displayed a tendency to have lower CD69 MFI, when exposed to the GSK3 inhibitor, independently of the presence or absence of IL7 (Figure 19.B).

To assess the response to IL-7 stimulation upon Wnt modulation, we evaluated the expression of related markers, including CD127 and Bcl-2 MFI and the percentage of CD25 expressing cells (Figure 19.C). The impacts found were very mild, as we observed a tendency for lower CD127 expression when in presence of the GSK3 inhibitor in the conditions without IL-7 stimulation (Figure 19.C). Moreover, there is also a tendency for a decrease in capacity of IL-7 to induce CD25 when the GSK3 inhibitor is added (Figure 19.C). No clear impacts were found with the modulation by DKK-1 (Figure 19.C).



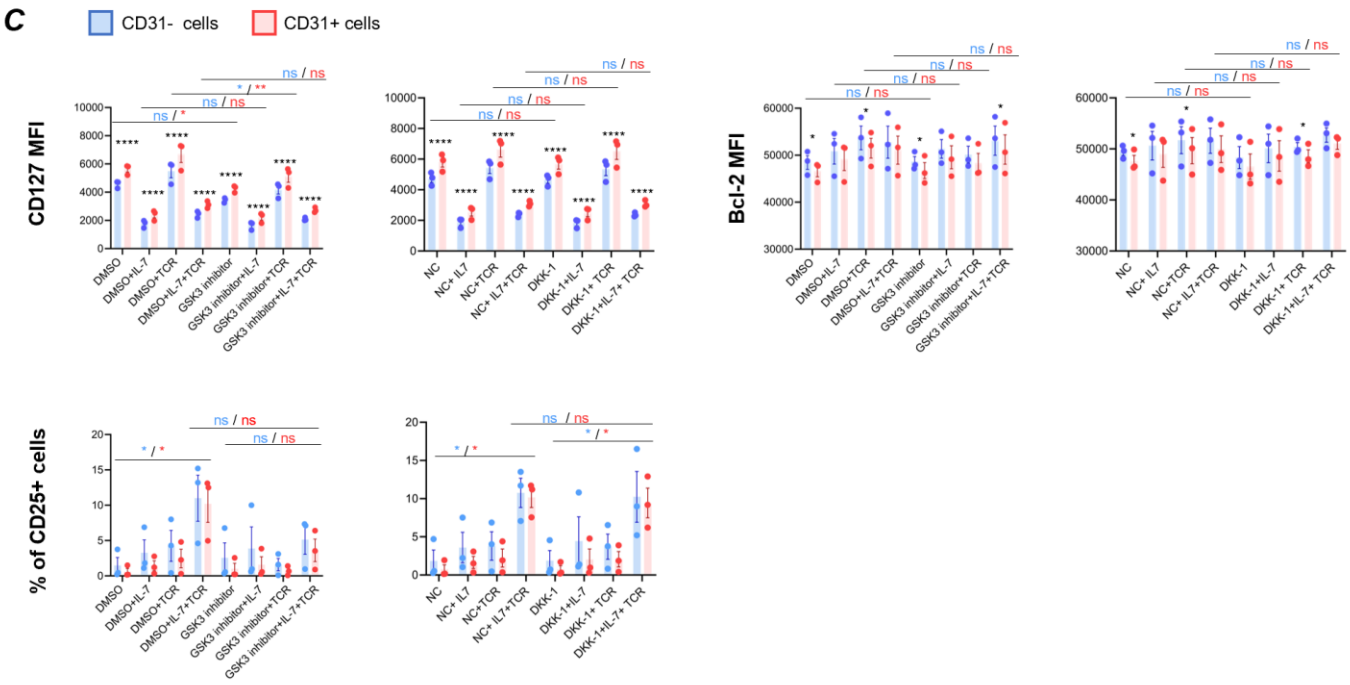


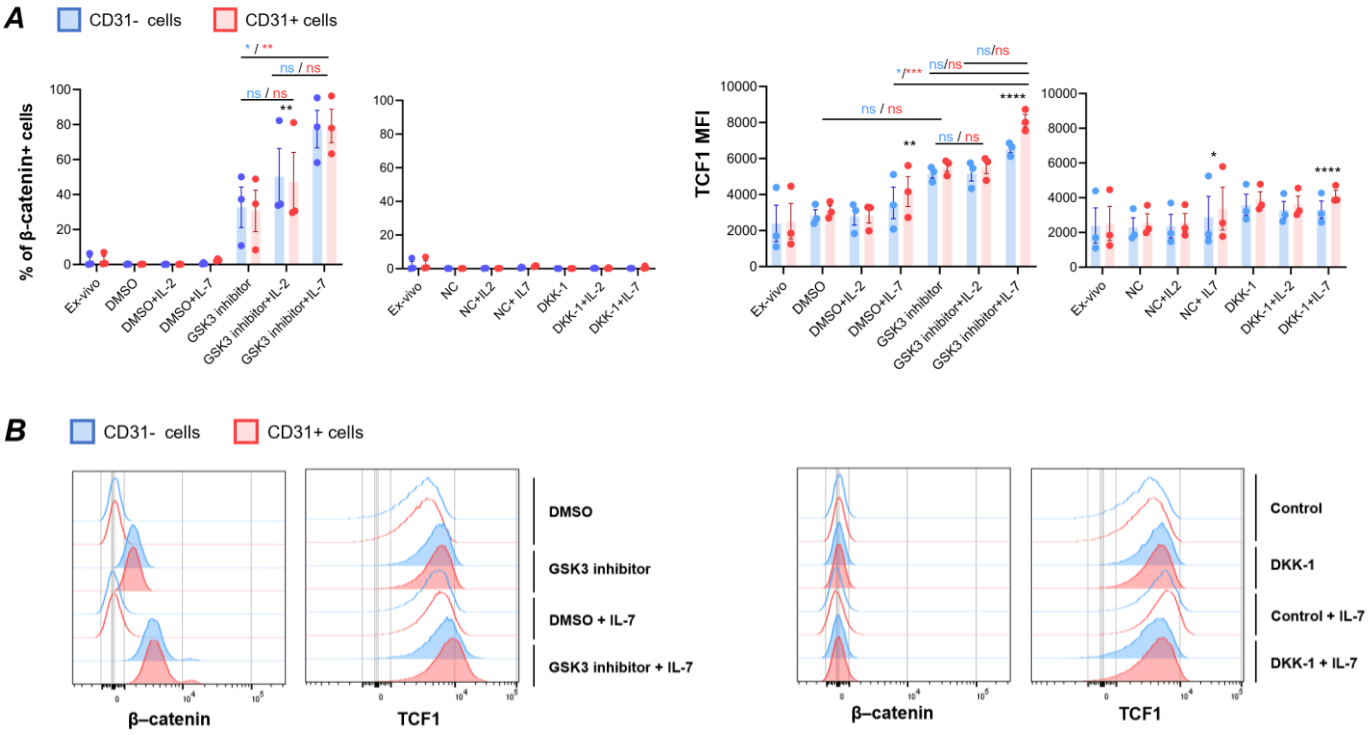
FIGURE 19: SHORT-TERM ALTERATIONS IN TCR AND IL-7 RESPONSE UPON WNT SIGNALLING MODULATION. (A) Quantification of the expression of β-catenin expressing cells and TCF1 MFI in the CD31-defined naïve CD4 Tconv subsets after the 3 hours cultures in the presence Wnt modulation and TCR and/or IL-7 stimulation. (B, Top) Percentage of CD40L and CD69-expressing activated cells and (down) measured MFI of the same markers within the CD31-defined activated cells. (C) Quantification of CD127 and Bcl-2 MFIs and percentage of CD25-expressing cells in the CD31-defined naïve CD4 Tconv subsets after the 3 hours cultures in the presence Wnt modulation and TCR and/or IL-7 stimulation. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on matched samples with two-way ANOVA and Sidak's multiple comparison (CD31neg vs CD31+ cells) and Tukey's multiple comparison tests (compare culture conditions). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001). GSK3 inhibitor: Wnt activator CHIR99021; DDK-1: Wnt inhibitor Dickkopf-1; NC: negative control; TCR: TCR-stimulation with CD3/CD28 beads.

6.2. Determining long-term IL-7 response alterations upon Wnt signalling modulation.

In the later 7 days stimulation time-point, we observed an increase in both β-catenin-expressing cells and TCF1 MFI, when the GSK3 inhibitor is added, without a significant difference when IL-2 is also present in the culture. However, when cells are stimulated with IL-7, this increase is substantial higher for both parameters (Figure 20.A and 21.B). Furthermore, we also found that, even though CD31 subsets show a similar percentage of β-catenin expressing cells, CD31+ naïve CD4 T_{convs} have a higher expression level of TCF1 than $CD31^{neg}$ cells, in the GSK-3 inhibitor + IL-7 condition (Figure 20.A and 20.B), suggesting a stronger impact in this subset. The modulation by DKK-1 does not show any relevant changes in both parameters for the established time point in this experiment, even in the presence of IL-2 or IL-7 stimulation (Figure 20.A and 20.B). Moreover, when assessing CD31 expression, neither the activation nor inhibition of Wnt signalling caused alterations in the percentage of CD31-expressing cells or CD31 MFI (Supplemental Figure 9).

To assess the response to IL-7 stimulation upon Wnt modulation, we quantified the expression of related markers, including CD127 and Bcl-2 MFI and the percentage of CD25 and Ki-67 expressing cells (Figure 20.C). After the 7 days culture, we also observed changes caused by the GSK3 inhibitor. This modulator, alone or in

presence of IL-2, did not show significant alterations in the IL-7-related markers. However, in IL-7 stimulated cells, we found a decrease in the downregulation of CD127 and in the Bcl-2 upregulation (Figure 20.C). These changes were accompanied by an important increase in the percentage of proliferating cells (Figure 20.C). Furthermore, while the described alterations caused by the GSK3 inhibitor, eliminate the intrinsic differences between CD31 subsets in the expression levels of CD127 and Bcl-2, we still observe a marginally higher percentage of Ki-67+ cells within the CD31+ subset (Figure 20.C). The upregulation of CD25 observed upon IL-7 stimulation is not affected by GSK3 inhibition, with the exception of a mild decrease in CD31^{neg} cells, which increases the difference between the CD31-defined subsets when the GSK3 inhibitor is added (Figure 20.C). The modulation via DKK-1 led to little measurable effects with only a mild effect in the fraction of CD25-expression upon IL-7 stimulation, lower when in presence of DKK1 (Figure 20.C).



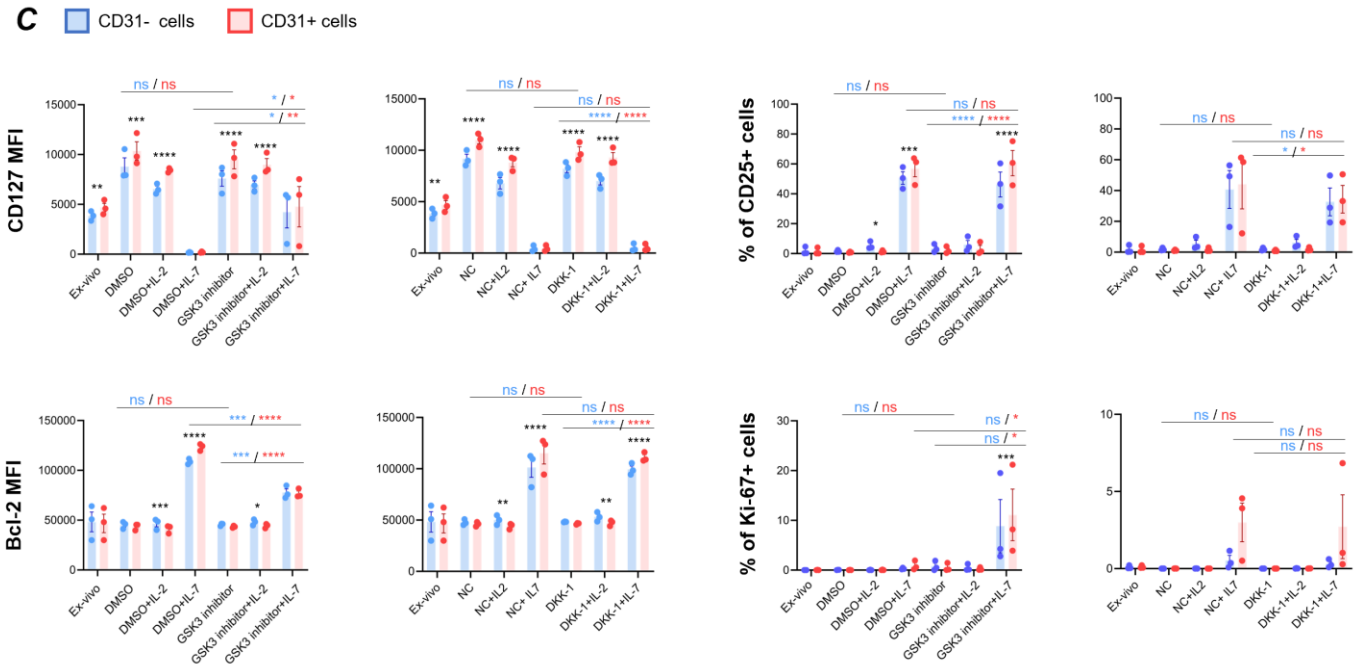


FIGURE 20: WNT MODULATION ALTERS NAÏVE CD4 T CELLS RESPONSE TO LONG-TERM IL-7 STIMULATION. (A) Quantification of the expression of β-catenin expressing cells and TCF1 MFI in the CD31-defined naïve CD4 Tconv subsets after the 7 days culture in the presence Wnt modulation and IL-2 or IL-7 stimulation. (B) Representative histograms displaying β-catenin and TCF1 expression within CD31-defined subsets upon long-term culture with Wnt signalling modulators and IL-7 stimulation. (C) Measured MFI of CD127 and Bcl-2 and percentages of CD25 and Ki-67-expressing cells within CD31-defined subsets after the 7 days culture in the presence Wnt modulation and IL-2 or IL-7 stimulation. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on matched samples with two-way ANOVA and Sidak's multiple comparison (CD31neg vs CD31+ cells) and Tukey's multiple comparison tests (compare culture conditions). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001). GSK3 inhibitor: Wnt activator CHIR99021; DDK-1: Wnt inhibitor Dickkopf-1; NC: negative control.

6.3. Phenotype of naïve CD4 T cells upon long-term IL-7 stimulation and Wnt modulation.

Besides the described alterations in IL-7 stimulation-related markers, we took advantage of the high-dimensional flow cytometry to more deeply evaluate impacts on long-term Wnt signalling modulation on the phenotype of naïve CD4 T_{convs}, especially when responding to IL-7 (Figure 21). While we found a tendency for a decrease, in the CD38 expression levels when cells are cultured with GSK3 inhibitor and IL-7, we also observed, in contrast, a substantial increase in the expression of the same marker when cells were exposed to both DKK-1 and IL-7. Additionally, both impacts were stronger in the $CD31^{neg}$ naïve T cell subset (Figure 21.A).

We also found that both IL-7 and GSK3 are able to increase the percentage of CD95-expressing cells and, when together, they have an additive effect, causing a significant increase not only in the percentage of CD95+ cells but also in the expression levels of this marker (Figure 21.B). This increase in the CD95 MFI is substantially higher in the CD31 expressing naïve T cell subset (Figure 21.B). The described effects were only observed in the longer 7 days time-point (see Supplemental Figure 8.B for the 3 hours time-point).

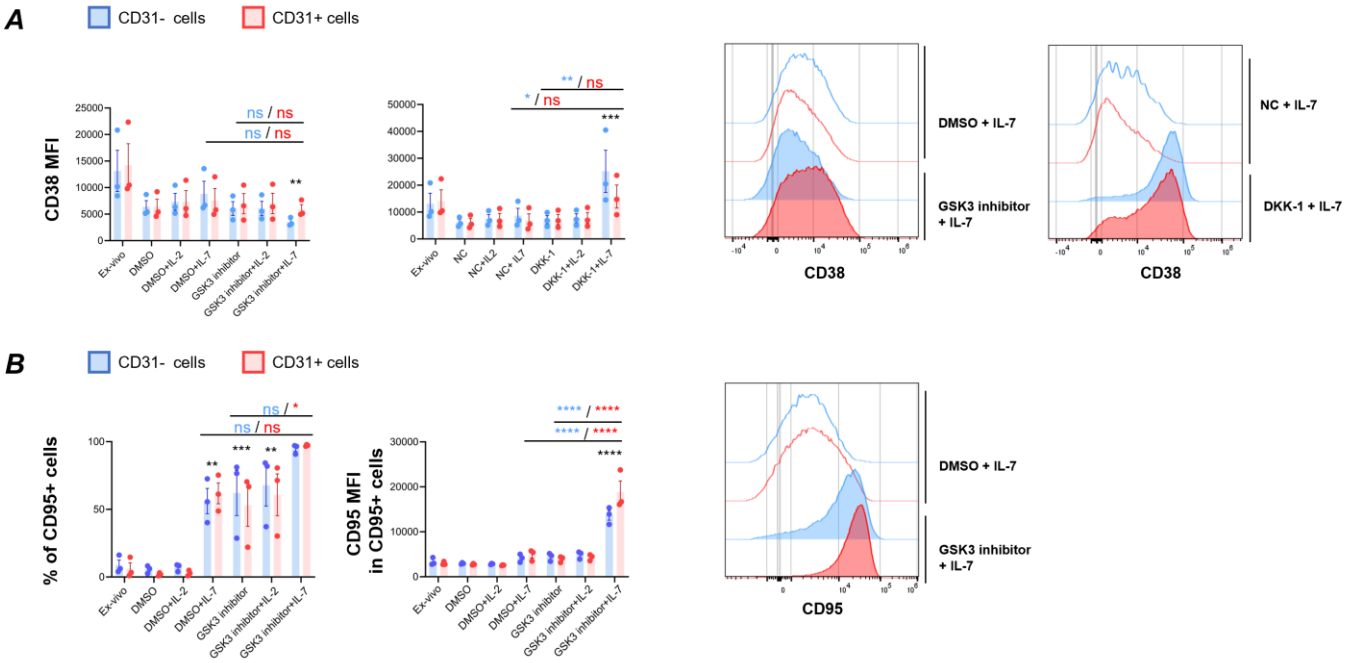


FIGURE 21. WNT MODULATION ALTERS DIFFERENTLY THE PHENOTYPE OF CD31-DEFINED NAÏVE CD4 T_{CONV} SUBSETS UPON STIMULATION WITH IL-7. (A, left) Measured CD38 MFI in the CD31-defined subsets upon long-term modulation of Wnt signalling with IL-2 or IL-7 stimulation and (A, right) representative histograms displaying CD38 expression after the 7 days culture with the Wnt modulators and IL-7 stimulation. (B, left) Quantification of the percentage of CD95-expressing cells and the CD95 MFI within the expressing cells in the CD31-defined subsets upon long-term modulation of Wnt signalling with IL-2 or IL-7 stimulation and (B, right) representative histogram of CD95 expression upon the 7 days culture in the presence of GSK3 inhibitor and IL-7 stimulation. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on matched samples with two-way ANOVA and Sidak's multiple comparison (CD31neg vs CD31+ cells) and Tukey's multiple comparison tests (compare culture conditions). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001). GSK3 inhibitor: Wnt activator CHIR99021; DDK-1: Wnt inhibitor Dickkopf-1; NC: negative control.

Finally, to better explore the phenotypical changes of the naïve CD4 T_{convs} upon the longer-term (7 days) modulation of the Wnt signalling and take full advantage of our large panel of markers (see Table 5), we also applied unsupervised analysis in this experiment. Here, we generated a UMAP on the concatenated events, with all conditions identifiable and with equal representation (Figure 22.A). Moreover, the conditions were also organized in a heatmap, to better display the relative expression of the markers (Figure 22.B and Supplemental Figure 10).

The overall impact of the stimulation conditions is clearly observed when considering the position of the different conditions in the UMAP projection (Figure 22.A, right). As expected from the previous results and from the lack of CD25 (IL-2Rα) expression by the naïve T_{conv} cells cultured, conditions with IL-2 stimulation are very similar to their corresponding modulation without cytokine stimulation. In opposition, conditions with IL-7 stimulation are clearly segregated based on the expression of CD127 and CD95 (Figure 22.A).

We also observed that DKK-1 has a similar phenotype to its negative control (NC), independent of the additional stimulation (Figure 22.A). The previously described increase in the expression of CD38, when added

together with IL-7 stimulation (Figure 21.A), is not apparent in the UMAP but it is shown in the heatmap (Figure 22.A e 22.B).

However, the phenotype of the cells modulated by the GSK3 inhibitor is visibly different from the respective controls (DMSO) and DKK-1 conditions, as shown in both the UMAP and heatmaps (Figure 22.A, 22.B and Supplemental Figure 10). Furthermore, the GSK3 inhibition, when together with IL-7 stimulation, shows a different phenotype from the GSK3 inhibition with IL-2 or no cytokine stimulation and the control with IL-7 stimulation (Figure 22.A and 23.B). This segregation is mainly explained by the markers associated to the activation of Wnt signalling, with the increase of β-catenin and TCF1 expression, but also with the increase of CD95 expression as observed previously[55] (Figure 21.B). The additional effects of IL7+ GSK3 inhibition, seen in the UMAP, are created by a higher expression of CD25, CD95 and Ki-67, when compared with GSK3 inhibitor with IL-2 or no stimulation, and the higher expression of β-catenin and CD127, when compared with the other IL-7 stimulated conditions (Figure 22.A). We additionally found that this condition (Wnt activation plus IL-7 stimulation) is also richer in cells expressing ICOS, CXCR3 and GATA3, as also shown in the heatmap (Figure 22.A and 22.B). Overall, these results clearly demonstrate cross-impacts of Wnt signalling associated molecules with the response of naïve CD4+ T cells to the homeostatic cytokine IL-7.

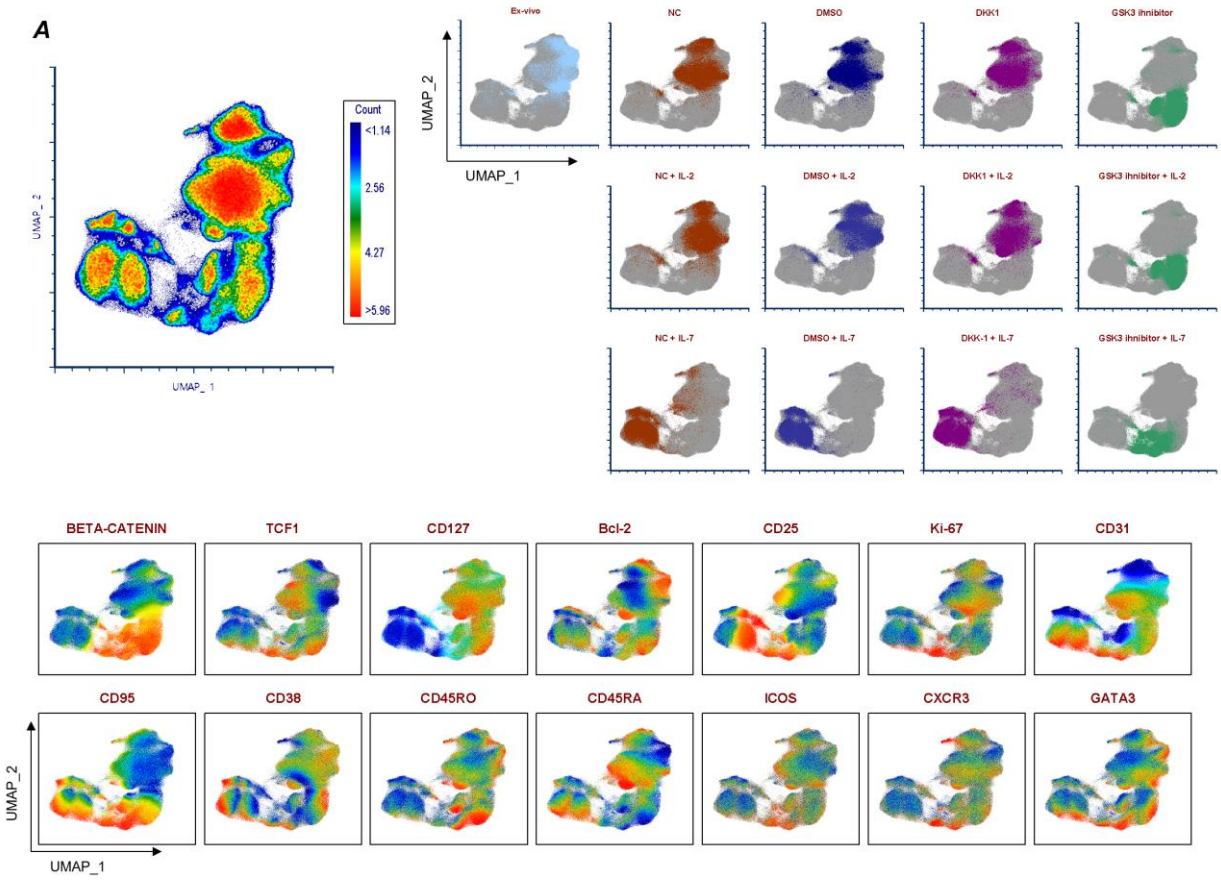


FIGURE 22: UNSUPERVISED ANALYSIS OF THE NAÏVE CD4 T_{CONVS} PHENOTYPE UPON LONG-TERM CULTURE WITH WNT MODULATION AND CYTOKINE STIMULATION. (A) Representative UMAP showing the phenotype of naïve CD4 Tconvs *ex-vivo* and after the 7 days culture with Wnt modulators, GSK3 inhibitor and DKK-1, and IL-2 or IL-7 stimulation with additional figures displaying (right) the events per culture condition and (under) the relative expression of the markers considered for this experiment. (B) Heatmap to compare the relative expression of the markers within naïve CD4 T cells across the culture conditions with Wnt modulation and IL-7 stimulation (see Supplemental Figure 10 for the additional IL-2 conditions). GSK3 inhibitor/GSK3i: Wnt activator CHIR99021; DDK-1: Wnt inhibitor Dickkopf-1; NC: negative control.

Discussion

7. Heterogeneity within the naïve CD4 T cell compartment

7.1. CD31-defined subsets show only subtle differences in phenotype.

At the start of this project, we used paired samples of blood and tonsils to better characterize the CD31-defined subsets within the naïve CD4 T cell compartment and investigate a possible link between these subpopulations and the Wnt/TCF1 axis and IL-7 signalling. When we firstly evaluated the expression of CD31 in the memory and naïve CD4 T cell populations, we found, as expected, that the majority of naïve T_{convs} and T_{regs} express CD31, while the memory CD4 T cells do not. However, T_{scms}, a rare memory population with a very similar phenotype to naïve cells, do not follow the expression pattern of either naïve or memory populations. This arises questions regarding the physiological generation of this small memory compartment. Since CD4 T cells lose the expression of CD31 upon TCR activation, it is interesting that CD4 T_{scms} retain a large fraction of the CD31 expression, possibly reflecting either the quality or the intensity of the activation signals received. As already referred in the introduction, the differentiation to T_{scms} is yet to be completely understood and most information originates from pharmacological induction to artificially generate this subset[23]. Further studies are needed to understand the molecular mechanisms that play a role on this subset generation.

While characterizing the CD31-defined subpopulations within the CD4 naïve T_{convs}, we did not find any clearly distinct differences between the phenotype of both. Overall, the two subpopulations are shown to only have different levels of expression of naïve or T cell-lineage markers. In the physiological context, these small differences might have an impact on how they respond to the environmental stimuli, possibly creating a distinct pre-disposition to a certain response or fate. When we investigated the expression of activation/homing markers, we found that $CD31^{neg}$ naïve CD4 T_{convs} have a higher percentage of CD69 in the tonsils. It is worth noting that, although CD69 is commonly known as an early-activation marker, it is equally involved in the lymphocyte retention in the SLOs, also in response to TCR stimulation[94]. Moreover, we also found that CXCR3-expressing cells, a marker shown to be associated with a "resting" early-memory subpopulation, has a higher percentage within the $CD31^{neg}$ naïve subset[75]. Thus, these results strengthen the hypothesis that $CD31^{neg}$ subset is more prone to respond to tonic TCR stimulation[3,83].

The analysis of IL-7-related markers expression, confirms the previously found discrepancy in the expression of CD127, which hints that CD31-expressing T_{convs}, which have higher levels of the IL-7 receptor, rely more on the homeostatic stimulation by IL-7[17]. However, the CD31-defined subsets show similar levels of Bcl-2 expression and Ki-67-expressing cells, which, according to previously published studies from the lab, are higher in the $CD31^{neg}$ naïve T cells [17]. It is possible that the results previously described are not seen here because of the small sample number. We also found the naïve CD4 T_{convs} to express TCF1, even though we could not detect β-catenin expression. It is possible that the naïve compartment is not responding to Wnt

stimulation in *ex-vivo* conditions and that this is a constitutive expression of TCF1, sustained in a Wnt-independent way, but further experiments are required to elucidate this issue.

Altogether, even though the CD31-defined subsets only show nuanced differences in their phenotype, these differences can be reflected in a distinct propensity when responding to stimuli. However, it remains unknown if these differences are maintained by CD31 signalling and, if so, by which molecular mechanism these differences are achieved. Mold et al. reported that CD31-defined subsets have a different level of basal expression of NF-kB, which, in turn, affects the response to IL-7 stimulation[2]. It is possible that the different expression levels of the reported markers can also be the result of a distinct expression level of NF-kB. Our experiments on the CD31 modulation (further discussed below) did not yield conclusive results, thus these questions remain.

7.2. The naïve CD4 T cell compartment presents different TCF1-defined subsets.

When we performed the unsupervised methodology to investigate CD31-associated subsets, we were surprised to find, instead, subpopulations clearly divided by different levels of expression of TCF1. Firstly, we saw that naïve T_{regs} have a lower expression of TCF1 than T_{convs}. In fact, TCF1 has been considered a negative regulator of Tregs, since it has shown to repress FOXP3 expression and inhibit the suppressive function of this subset[99,100]. However, given TCF1's importance to maintain the naïve T cell phenotype, it is plausible that it is only downregulated to low levels of expression to also guarantee FOXP3 expression and, thus, maintain T_{regs} identity[100].

Within the T_{convs} we found two distinct subsets defined by a different expression level of TCF1. We first found a subset of CD31+ naïve CD4 T cell where we could not detect TCF1. We found it contradictory, since TCF1 is important to maintain the naïve phenotype and it is only downregulated during effector differentiation[69]. Moreover, the only additional clue we found related to this subset is its average lower level of Bcl-2 expression. Based on studies on CD8 T cell exhaustion, a state of cell dysfunction, TCF1 expression has shown to be positively correlated with a better effector function and to promote the c-Myb/Bcl-2 survival axis[101,102]. Therefore, it might be possible that the TCF1 negative cells that we observed may be similarly entering a state of exhaustion, senescence or, simply put, a state of dysfunction[66,101,102].

On the other side, we also found, exclusively on tonsils, a subset of CD69-expressing T_{convs} with a higher expression level of TCF1. This subset was further characterized as having a lower expression of CD127 and higher expression of CD38 and ICOS. As already discussed, CD69 is a marker of early T cell activation but also of tissue retention, usually upregulated in response to TCR stimulation[94]. In this context and based in the low expression of the IL-7 receptor, it is possible that this subset, present in the tonsil, represent cells responding to IL-7. However, it important to note, based on published studies and our own results, that IL-7 stimulation, by itself, does not promote CD69 upregulation[103]. Similarly, ICOS and CD38 are not upregulated by IL-7

stimulation (Figure 21 and Supplemental Figure 11). Therefore, this phenotype is not induced by the response to IL-7. Because these are markers usually studied in the context of T cell activation, it is difficult to hypothesize on their function in this subpopulation of naïve CD4 T cells, but in alternative, these could represent recently activated T cells not yet displaying changes in naïve/memory markers.

Another possibility can be that this subset is, in fact, represents precursors of follicular helper T cells (T_{fh}), an effector CD4 T cell subset involved in the development of the B-cell response. The combined expression of $CD38^{high}$ $ICOS^{high}CD127^{low}$ is used to identify activated T_{fh} cells and, additionally, the upregulation of CD69 might be need to retain this subset in the SLOs[104,105]. Moreover, TCF1 is one of transcription factor known to be involved in the differentiation to this subset[106]. However, this population does not express the characteristic markers of T_{fh} cells, PD-1 and CXCR5[105].

8. In search of CD31-specific effects on short-term TCR stimulation.

In this project, we were aiming to better establish the function of CD31 signalling in the short-term responses to tonic-TCR stimulation and IL-7 or Wnt signalling pathways. Based on our current knowledge from the literature, CD31 has inhibitory function over the TCR signalling[83]. Therefore, we were expecting that, at least, the modulation of its signal would result in changes on the TCR activation of the CD31+ naïve CD4 T cells. In normal conditions, we found mild to no differences between the CD31 subsets in the parameters considered to study T cell activation. However, out of 3 chosen modulators of the CD31 signalling, only one, SHP099, showed an impact on the analysed parameters. Assuming that the CD31 modulators were effective, it is possible that their effects were too subtle to be observable in the designed experiments. However, it is also questionable if the modulators performed to the expectations. Although we attempted to titrate effects prior to performing the experiments, the results from these preliminary tests were not clear. Indeed, it may be harder to obtain an efficient blocking of CD31 signalling when the large majority of the cells in culture express the CD31 and its heterophilic ligand, CD38, thus, creating an environment abundant on CD31 engagement. Increasing significantly the dosage of the CD31 blocker as well as including CD38 blockage might have yielded better results. Moreover, regarding the modulation by the agonistic CD31 peptide, the fact that in some conditions the capacity to detect CD31 expression is hampered by the presence of the agonist leads to results which are harder to interpret, and experiments with sorted CD31+ cells may be required to address our questions. Given the many unexpected factors that might impact on the experimental CD31 modulation, in future optimizations, we aim to further confirm it by measuring the phosphorylation levels of SHP-2.

Regarding the modulation with SHP099, we were aiming to hinder the CD31's inhibitory function on TCR signalling pathway and, thus, we were predicted to observe an increase on TCR activation. However, upon TCR stimulation, we found a small decrease in the percentage of activated cells and an impactful decline on the

expression levels of the activation markers, CD40L and CD69. Moreover, even SHP099 effects did not show to be CD31-specific, making it difficult to associate any found observation to the function of CD31. The specific role of SHP-2 in the TCR signalling pathway is yet to be completely understood, however, there are studies that similarly report a decline on TCR activation, when SHP-2 function is abrogated in T cells[107,108]. Thus, it is more likely that the described observations are the result of the specific SHP-2 inhibition, rather than the expected inhibition of CD31-related SHP-2 function.

The only CD31-specific alteration we found was related with CD127 expression. After the short-term culture, CD127 was always found to be differently expressed in the CD31-defined subset, even in the presence of TCR and/or IL-7 stimulation. Moreover, when modulated with SHP099, we found that CD127 expression decreases in CD31+ naïve CD4 T cells, in the presence of TCR stimulation only. Nevertheless, we did not find the modulation to be impactful in the downregulation of CD127 upon IL-7 stimulation. Based on these results, and allowing for some degree of speculation, it might be possible that CD31 is involved in the regulation of CD127 localization in the membrane, therefore, explaining the fast internalization without IL-7 stimulation. Overall, further experiments are still required to elucidate these aspects.

9. Wnt Signalling Modulation and the response of naïve CD4 T cells to IL-7.

9.1. Short-term Wnt modulation with GSK3 inhibitor and response to TCR and IL-7 stimulation.

Given the importance of Wnt/TCF1 in T cell-related processes and our results describing TCF1-defined heterogeneity within the naïve CD4 compartment, we wanted to investigate further and confirm whether Wnt/TCF1 signalling impacted the response to homeostatic stimuli, including the early response to TCR and IL-7 stimulation.

When we first searched for indicators of modulation of the Wnt pathway, there was no evidence of change in the expression of β-catenin and TCF1 from both modulators after 3 hours. We found, however, that GSK3 inhibition had an impact on TCR and IL-7-related markers. After TCR stimulation, we observed a lower upregulation of CD69, without, however, alteration of the number of activated naïve T cells. Regarding the IL-7-related markers, we observed a lower expression of CD127, in conditions without IL-7, and a decrease in the CD25-expressing cells induced by the combined stimulation of TCR and IL-7 signalling. Since we found no clear evidence of Wnt activation, it possible that these effects resulted from the GSK3 inhibition but are not Wnt-related. There are no evidences of GSK3 being involved in the upregulation of CD69[109]. However, it is known that GSK3 is part of IL-7R signalling pathways, thus, explaining the alterations observed on CD127 and CD25 expression. As referred in the introduction, GSK3 is also part of a complex, in combination with FOXO1 and FOXO-3a, that regulates the expression of IL-7 related genes, including the expression CD127 (see Figure 2)[33,43].

9.2. Wnt modulation and GSK3 inhibitors strongly impact naïve CD4 T cell the response to IL-7.

Similarly to the early response to TCR and IL-7 stimulation, we also aimed to investigate the impact of the Wnt/TCF1 axis on the long-term response to IL-7.

The substantial increase in frequency of β-catenin expressing cells and TCF1 expression levels, confirmed the desired modulation in the presence of the GSK3 inhibitor. This increase was further promoted by IL-7 stimulation, which, additionally, induced a higher upregulation of TCF1 in the CD31-expressing cells. Therefore, IL-7 stimulation is able to strengthen Wnt signalling, once this pathway is activated. Additionally, we also found, once more, evidence that show CD31-expressing cells are more responsive to IL-7 signalling. We did not find evidence of β-catenin or TCF1 modulation by DKK-1, even in the presence of IL-7 stimulation. It is possible that DKK-1 did not have any effects because the Wnt pathways, itself, is not activated, and the existing expression of TCF1 in naïve CD4 T cells is not constitutively maintained by Wnt but we cannot exclude that higher concentrations of DKK-1 are required in this setting to detect changes in these parameters.

In the long-term response to IL-7, we found evidence that can be interpreted as GSK3 inhibitor hindering the response to IL-7, as we observed a decrease on the CD127 downregulation and Bcl-2 upregulation. In opposition, we also found that GSK3 inhibition significantly increases the frequency of proliferating cells, especially in CD31-expressing cells. Given the GSK3 inhibitor's decrease in the CD127 downregulation and the role of GSK3 in the regulation of IL-7R signalling, it is possible this mechanism is affected, allowing the cells to continue to respond indefinitely to IL-7, thus, explaining the substantial increase in the proliferation[33,43]. This condition could potentially be optimized to promote specific expansion of CD31-expressing naïve CD4 T cells. However, it is possible that not all effects observed with the GSK3 inhibition are dependent on the Wnt pathway, even if we could indeed observe a clear increase on β-catenin. Interestingly, the described effects after the 3 hours in CD127 and CD25 expression, are found in the opposite direction, hinting to different mechanisms being impacted on at early and late time points. Further testing with different Wnt signalling activators, including physiological ligands that do not interfere directly with GSK3 function, should be performed. Regarding the modulation by DKK-1, in opposition to what has been suggested by Kared et al. in induced CD4 T_{scms}, we did not find clear impacts on these parameters use to evaluate the response to IL-7 stimulation[55].

Besides these markers directly related to the IL-7 response, we also found that Wnt modulation alters the expression of other markers. We found CD38 expression in $CD31^{neg}$ naïve CD4 T cells to be altered by both Wnt modulators, exclusively in presence of IL-7. Indeed, while we observe a decrease when we add GSK3 inhibitor to the culture, there is, in opposition, an increase in CD38 expression, in the presence of DKK-1. These effects, in opposite directions, indicate that this modulation of CD38 expression is Wnt-dependent. DKK-1, physiological inhibitor of the canonical Wnt pathway, increases systemically with age, and, according to Kared

et al., is positively correlated with age-related impairments on the homeostasis of T_{scms}[55]. Similarly, CD38 is also involved in aging-associated immune-dysfunction due to its role in the metabolism of NAD+[110,111]. Therefore, it is possible that, in the physiological context, DKK-1 promotes "immune-aging" of the naïve CD4 T cell compartment by promoting CD38-related metabolic dysfunction.

We also observed, as expected, a substantial increase in CD95-expressing cells upon modulation by GSK3 inhibitor or IL-7 stimulation. When combined, the majority of the cultured naïve CD4 T cells were found to express CD95 and it even resulted in a significant increase of this marker's expression within positive cells. This effect, similarly to the increase in proliferation, might be result of the continuous response to IL-7, due to GSK3 inhibitor hindering the IL-7's negative feedback. As already discussed, CD95 has been described as marker to identify T_{scms} and Wnt activation has been reported to generate T_{scm} cells[19,23,55]. Even though the cultured cells do maintain the expression of several naïve markers, it is not clear whether these cells are real T_{scms} or whether these conditions will result in further differentiation of the naïve CD4 T cells. Further testing on these cells' function and stability of phenotype should be performed to assess if they, indeed, are T_{scm}.

Conclusion and Future Perspectives

In conclusion, our *ex vivo* characterization of CD31-defined naïve subsets revealed a different expression of naïve T cell related markers, activation/homing molecules and IL-7 receptor. However, there wasn't evidences of a correlation between CD31-defined subsets and Wnt-related markers. Further analysis using unsupervised methodologies revealed that the compartment of naïve CD4 T cell is heterogenous and can be subdivided based on TCF1 expression. In fact, we described the existence of a subpopulation of $TCF1_{neg}Bcl\text{-}2_{low}$ naïve CD4 T_{convs}, in both blood and tonsil, and another subpopulation, present only in the tonsils, characterized by the high expression of CD69 and TCF1 and low expression of CD127. Further characterization of these subsets should be performed to better understand their identity and additional information will also be obtained by integrating the protein expression data with single cell transcriptomics of human naïve CD4 T cells.

Following in the *in vitro* experimentation of CD31 modulation, given the unexpected experimental factors, the results were inconclusive and did not reveal clear CD31-specific effects. We are aiming to further optimize the experimental design, so that we can find supporting evidence or rule out a role of CD31 expression on the homeostasis of naïve CD4 T cells. More efficient CD31 blockers and possibly the addition of CD38 blockers may help to advance in these aspects.

Finally, our experiments on the modulation of the Wnt signalling pathway suggest the existence of a bidirectional crosstalk between Wnt and IL-7 signalling. Moreover, the combined modulation of Wnt pathways and Il-7 stimulation were associated with phenotypical changes, mainly in the expression of CD95 and CD38, which, in the physiological context, might have an impact of the naïve CD4 T cell compartment. Furthermore, CD31-defined subsets were found to respond differently, namely in respect to TCF1, CD38, CD95 and Ki-67 expression levels, upon the modulation. We aim to implement additional experiments, especially with Wnt-specific ligands, to better understand the interaction between the two signalling pathways and to optimize its effects. It is also important to not lose track of the GSK3 inhibitor mediated effects, as their impact on the response to IL-7 is relevant to elucidate on the exact molecular mechanism involved here. Due to the strong impacts observed and the relevance of the naïve and T_{scm} subsets, harnessing these mechanisms will provide opportunities in the future design of optimized protocols to preserve or modulate these cells in patients or immunocompromised individuals and to improve protocols in T cell-based immunotherapies.

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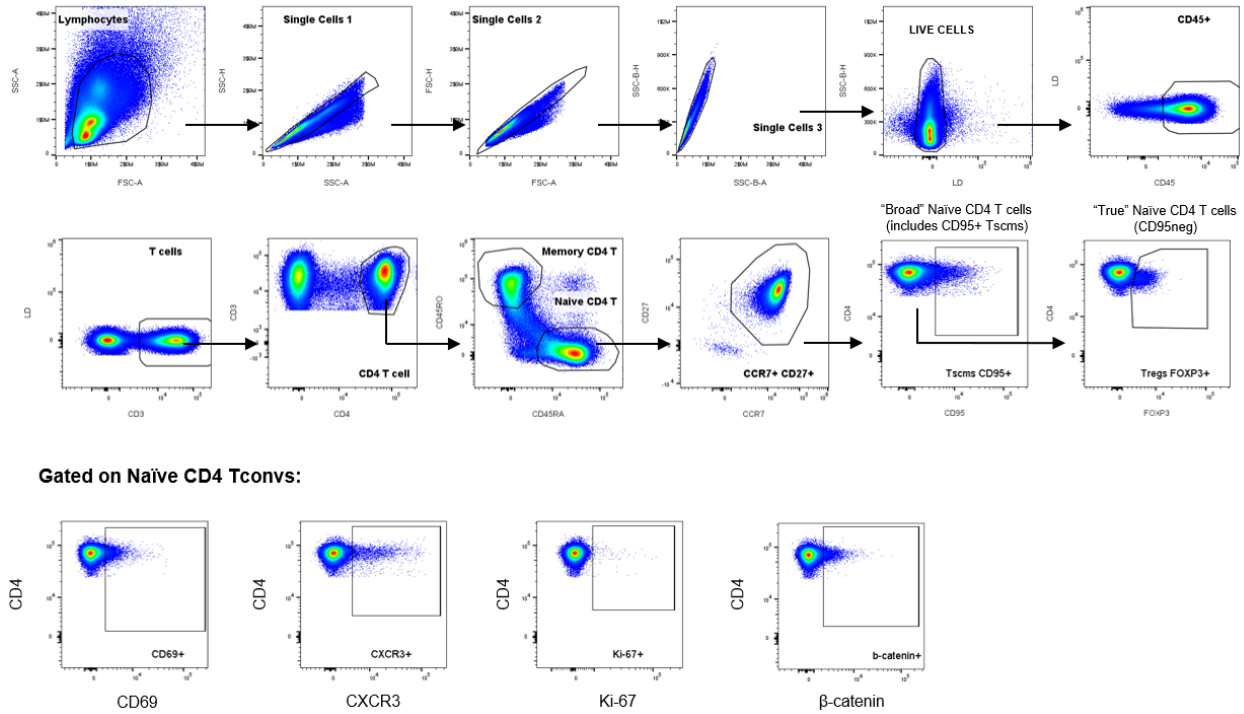
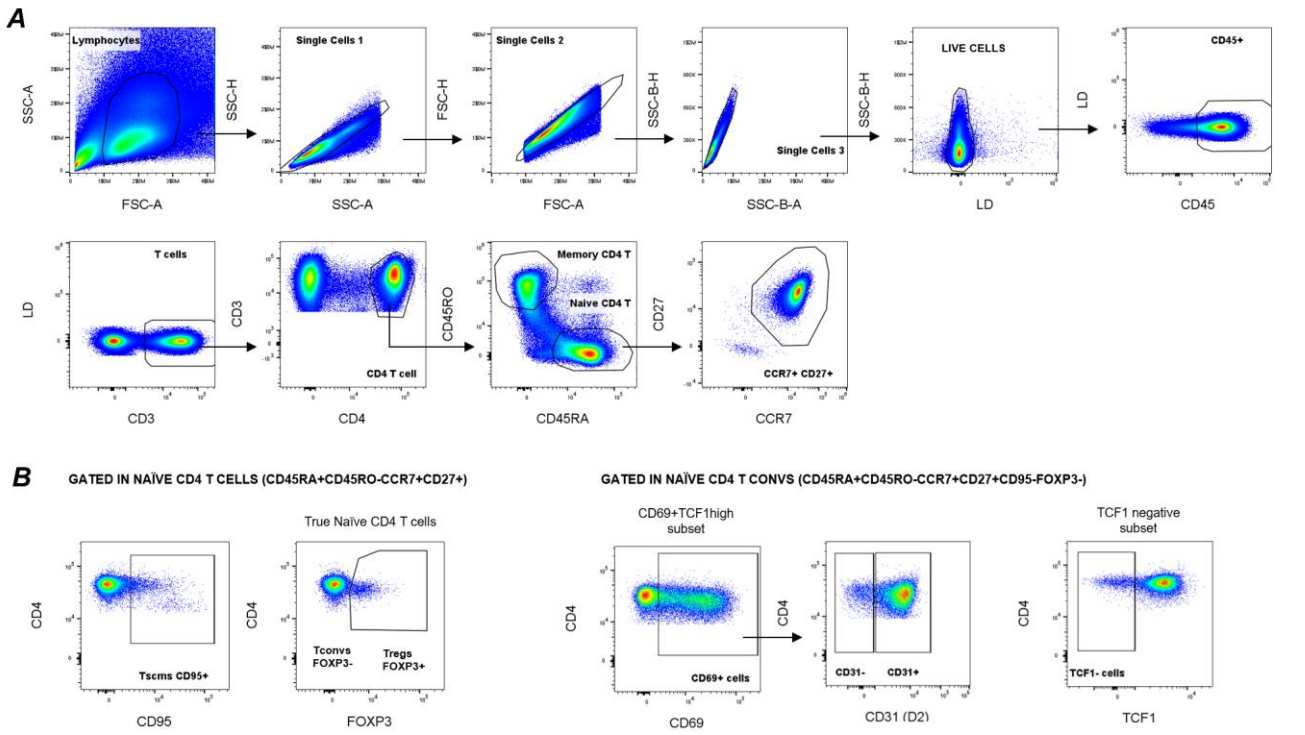
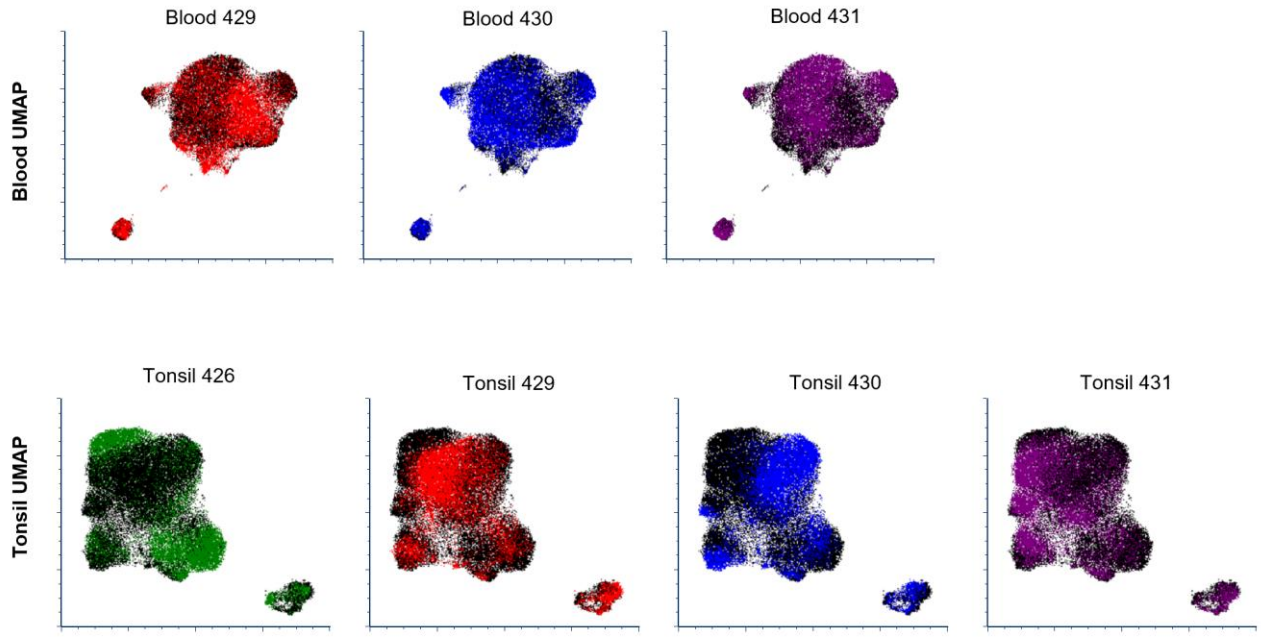
Supplemental Figures



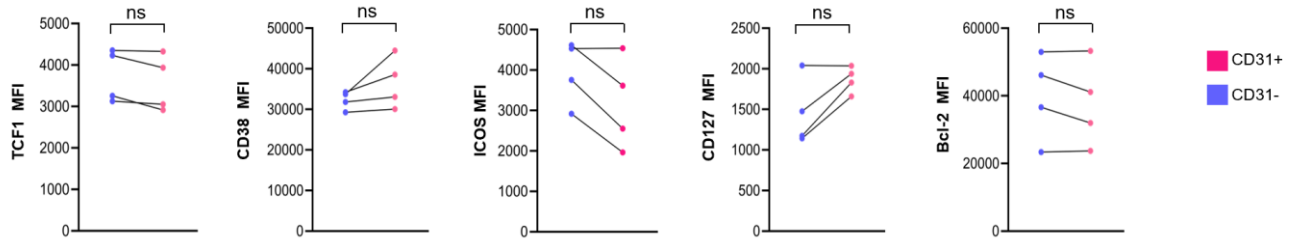
SUPPLEMENTAL FIGURE 1: COMPLETE GATING STRATEGY APPLIED ON SUPERVISED ANALYSIS ON THE PHENOTYPICAL CHARACTERIZATION OF THE CD31-DEFINED NAÏVE CD4 T CONV SUBSETS. (First two lines of plots) Gating strategy to identify naïve CD4 T cell population and subpopulations naïve CD4 Tconv and Treg. (A. third line of plots) Gating applied to quantify the percentage of CD69, CXCR3, KI-67 and β-catenin expressing cells in the CD31-defined subsets within naïve CD4 Tconvs.



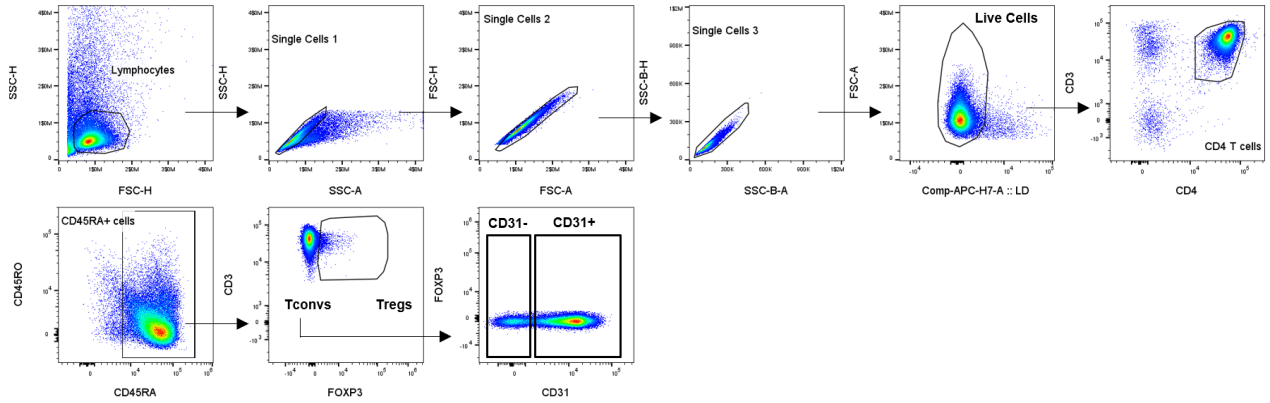
SUPPLEMENTAL FIGURE 2: COMPLETE GATING STRATEGY FOR UNSUPERVISED ANALYSIS AND SUPERVISED CONFIRMATION OF TCF1-DEFINED SUBPOPULATIONS PHENOTYPE. (A) Gating strategy applied to export "Broad" naïve CD4 T cells (CD4+CD45RA+CD45RO-CCR7+CD27+) for further unsupervised analysis. (B) Additional plots applied to identify the main TCF1-defined subpopulations (Tregs, TCF1 negative Tconvs and CD69+TCF1high Tconvs) within the naïve CD4 T cell compartment.



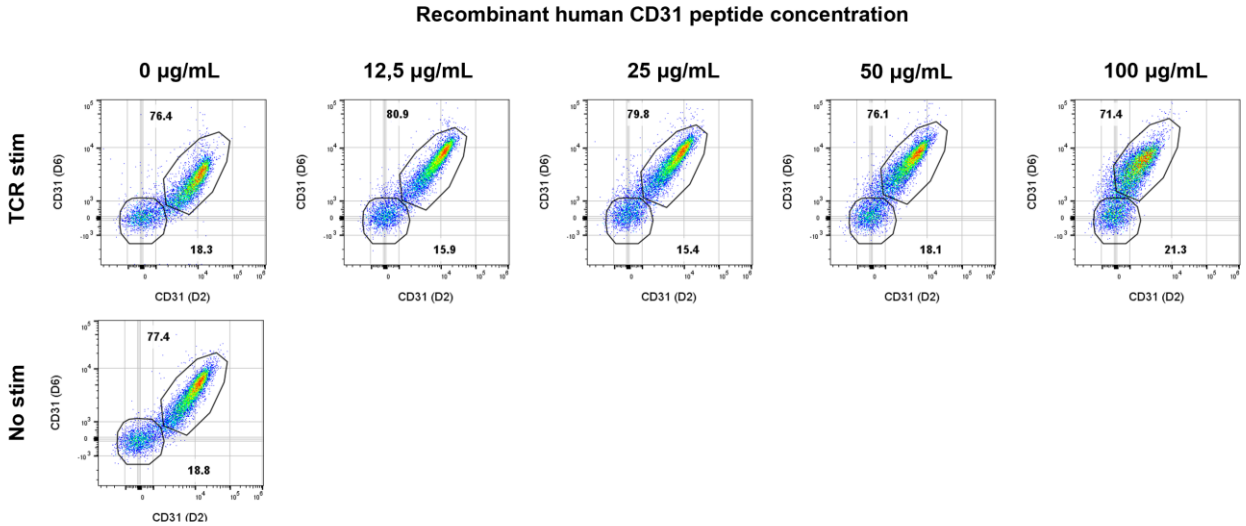
SUPPLEMENTAL FIGURE 3: INDIVIDUAL CONTRIBUTION OF THE CONSIDERED SAMPLES USED TO BUILD THE UMAP. Overlay of the events corresponding to each sample in the Blood and Tonsil UMAP.



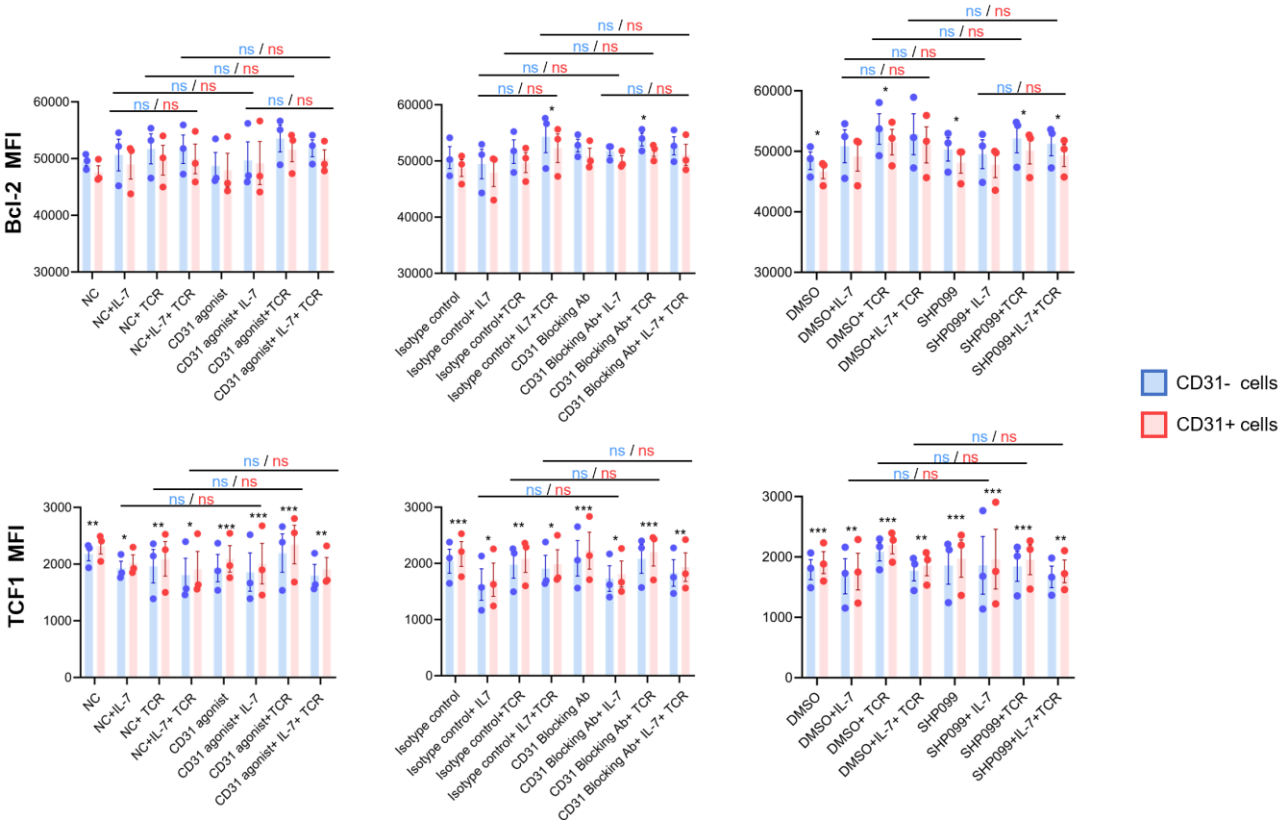
SUPPLEMENTAL FIGURE 4: CD31-DEFINED SUBSETS WITHIN CD69-EXPRESSING NAÏVE CD4 T_{CONVS} DO NOT SHOW DIFFERENTIAL EXPRESSION OF MARKERS THAT CHARACTERIZE THIS SUBPOPULATION. Quantification of TCF1, CD38, ICOS, CD127 and Bcl-2 MFIs in CD31-defined subsets within the CD69+ naïve CD4 Tconvs. Individual samples are depicted. Statistical analysis was performed on paired samples with paired T tests. The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001).



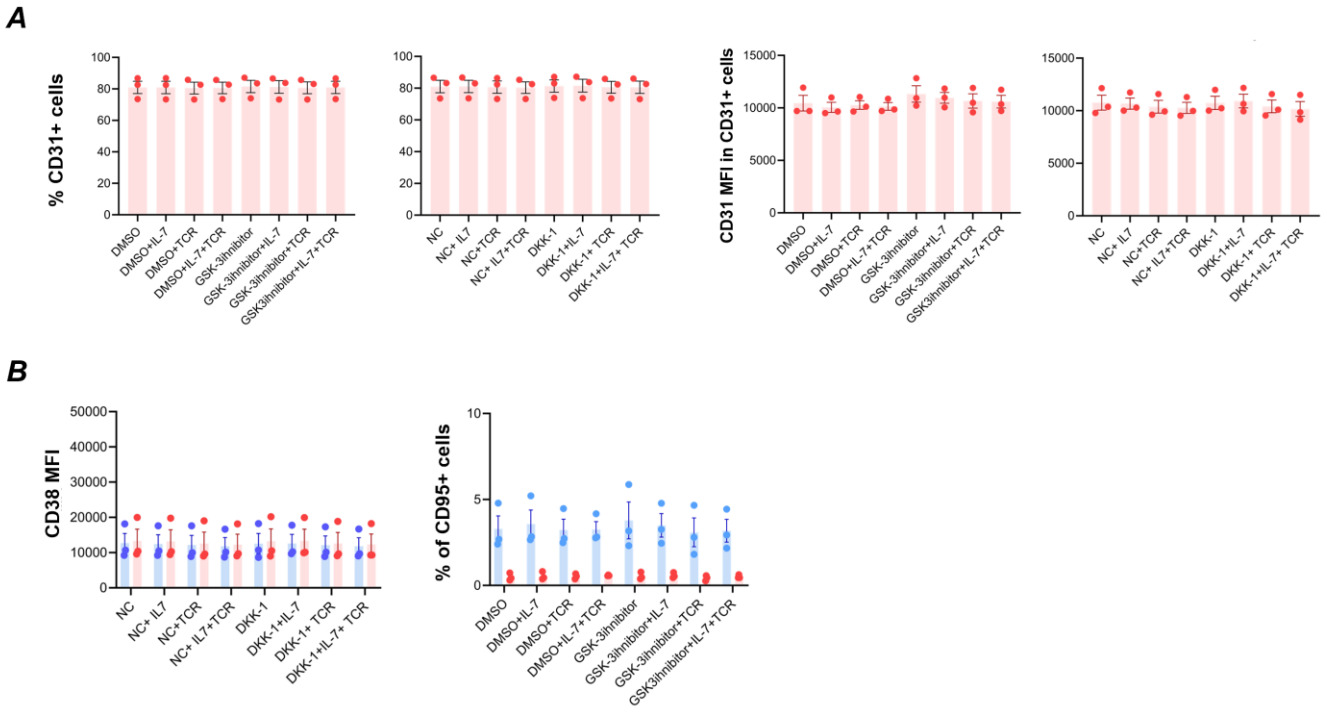
SUPPLEMENTAL FIGURE 5: COMPLETE GATING STRATEGY APPLIED FOR SUPERVISED AND UNSUPERVISED ANALYSIS OF SHORT-TERM AND LONG-TERM MODULATION OF CD31 AND WNT SIGNALLING. Gating strategy applied to identify naïve CD4 Tconvs and the CD31-defined subsets in the 3 hours culture in the presence of CD31 modulators and the 3 hours and 7 days culture in the presence of the Wnt modulators.



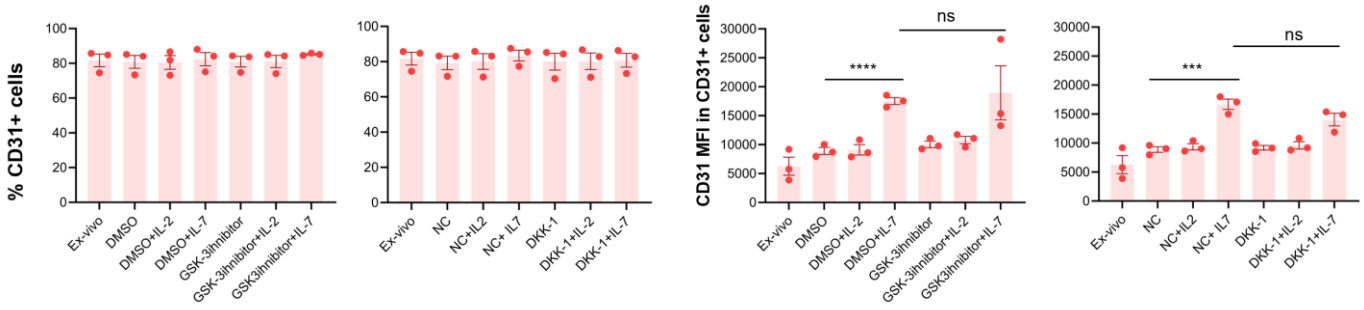
SUPPLEMENTAL FIGURE 6: CD31 DOMAIN 2 AND 6'S RECOGNITION UPON CD31 MODULATION WITH AGONIST CD31 PEPTIDE. Identification of CD31 subsets, based on the combined expression of domains 2 and 6 of CD31, upon a 3 hour modulation with increasing concentrations of CD31 agonist peptide, in the presence of TCR stimulation (1:8 beads to cells ratio).



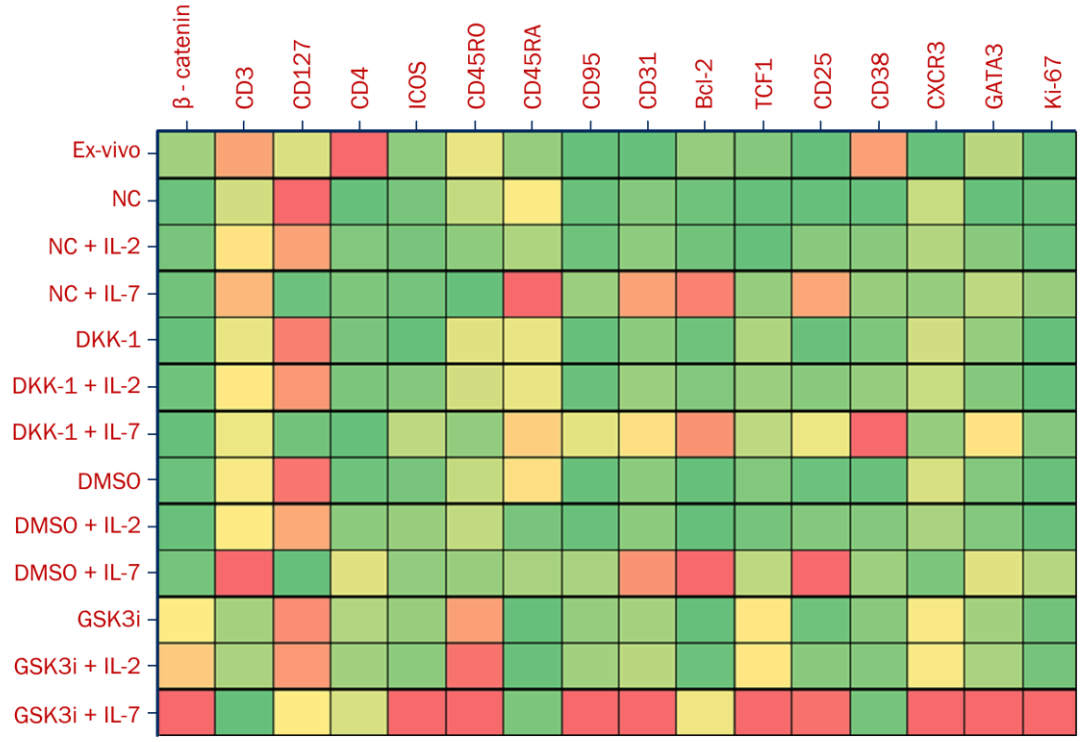
SUPPLEMENTAL FIGURE 7: BCL-2 AND TCF1 EXPRESSION UPON CD31 MODULATION AND SHORT-TIME TCR STIMULATION. Measured MFI of Bcl-2 and TCF1 within the CD31-defined naïve CD4 Tconvs, after the 3 hours culture with CD31 modulators (CD31 agonist peptide, anti-CD31 blocking Ab and SHP099) in the presence of TCR and/or IL-7 stimulation. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on matched samples with two-way ANOVA and Sidak's multiple comparison (CD31neg vs CD31+ cells) and Tukey's multiple comparison tests (compare culture conditions). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001). NC: negative control; CD31 agonist: recombinant human CD31 peptide; CD31 blocking Ab: anti-CD31 blocking antibody; SHP099: SHP-2 selective inhibitor; TCR: TCR-stimulation with CD3/CD28 beads



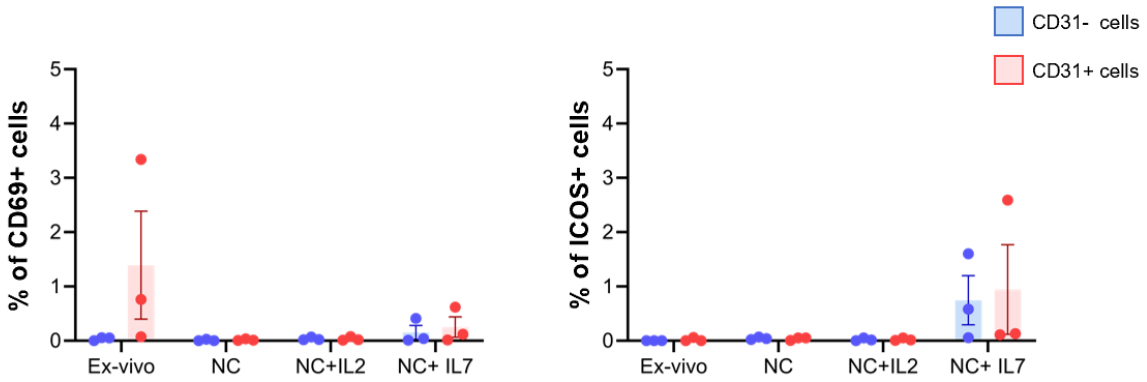
SUPPLEMENTAL FIGURE 8: SHORT-TERM WNT MODULATION DOES NOT INDUCE SIGNIFICANT ALTERATIONS IN THE PHENOTYPE OF NAÏVE CD4 T CELLS. (A) Quantification of the percentage of CD31-expressing cells and of the CD31 MFI within the CD31-expressing population upon 3 hours culture with Wnt modulators (GSK3 inhibitor and DKK-1) and TCR/IL-7 stimulation. (B) Quantification of CD38 MFI and percentage of CD95-expressing cells within CD31-defined naïve CD4 T conv subsets upon 3 hours culture with Wnt modulators and TCR/IL-7 stimulation. GSK3 inhibitor: Wnt activator CHIR99021; DDK-1: Wnt inhibitor Dickkopf-1; NC: negative control; TCR: TCR-stimulation with CD3/CD28 beads.



SUPPLEMENTAL FIGURE 9: CD31 EXPRESSION AFTER LONG-TERM CULTURE IN THE PRESENCE OF WNT MODULATION AND IL-7 STIMULATION. Quantification of the percentage of CD31-expressing cells and of the CD31 MFI in the CD31-expressing population upon 7 days culture with Wnt modulators (GSK3 inhibitor and DKK-1) and IL-2 or IL-7 stimulation. Individual samples, mean and standard error of mean are depicted. Statistical analysis was performed on matched samples with two-way ANOVA and Sidak's multiple comparison (CD31neg vs CD31+ cells) and Tukey's multiple comparison tests (compare culture conditions). The results presented follow the GraphPad p-value style (ns, p>0.05; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001). GSK3 inhibitor: Wnt activator CHIR99021; DDK-1: Wnt inhibitor Dickkopf-1; NC: negative control



SUPPLEMENTAL FIGURE 10: COMPLETE HEATMAP DISPLAYING MARKERS EXPRESSION PER CULTURE CONDITION IN THE LONG-TERM CULTURE WITH WNT MODULATION.
GSK3i: Wnt activator CHIR99021; DDK-1: Wnt inhibitor Dickkopf-1; NC: negative control.



SUPPLEMENTAL FIGURE 11: EXPRESSION OF CD69 AND ICOS UPON LONG-TERM CULTURE WITH IL-7 STIMULATION.
Quantification of the frequency of CD69 and ICOS-expressing cells upon 7 days culture with IL-2 or IL-7 stimulation.
NC: negative control