2023

Understanding the Journey of Human Prenatal CD4+ PLZF+ T Cells to the Small Intestine

Tomi Oyedeji Olaniyan

Understanding the Journey of Human Prenatal CD4+ PLZF+ T Cells to the Small Intestine

Tomi Oyedeji Olaniyan

Department of Neuroscience, W.M. Keck Science Department

Claremont McKenna College

KS190 : Senior Thesis

November 18, 2022

Abstract:

The fetal immune system has a variety of needs that differ from those of the adult immune system. One such difference is fetal T cell composition and how they migrate from the thymus to peripheral lymphoid organs and mucosal tissue such as the small intestine (SI). Previous research shows that the majority of memory T cells in the prenatal SI are CD4+ PLZF+ T cells but the mechanisms involved in the homing of these cells in the small intestine have not yet been determined. In our experiment, I found evidence to support the claim that IL-7 drives the acquisition of the memory phenotype in this subpopulation of naive T cells within 2-3 days. Additionally, we saw successful upregulation of other markers necessary for the successful homing of T cells in the gut such as the adhesion molecule - a4b7 and the chemokine CXCR4 within 2-3 days of IL-7 stimulation. By understanding the migration and accumulation of memory T cells in the SI, we might be able to reconstitute fetal immune systems that are lacking in this population and prevent the harmful effects of a deficient immune system.

Introduction

Our bodies are constantly on defense, against a never-ending stream of pathogens, viruses and sometimes even against our own cells. The study of immunology examines the mechanisms that help keep us healthy by doing everything from maintaining the homeostasis of our body to defending the body from foreign germs and pathogens. However, failure of our immune system to respond appropriately to invading pathogens, including extreme immune responses to our own self antigens, could lead to major adverse effects on human health and functionality. The ability to mediate one's immune response becomes increasingly more pressing in fetal immune systems, which quickly encounter large amounts of new pathogens for the first time. One major way in which human bodies can do this is through the accumulation of memory cells, which are able to remember previously encountered pathogens and help us fight them off quickly. However, the fetal immune system has differing types of memory to adults. In this thesis, I will be investigating the use of the cytokine IL-7 to improve migration of CD4+ PLZF+ T cells to the gut and activate them into memory T cells, a subsect of memory cells specific to fetal immune systems.

The immune system can be understood through two main categories - innate immunity and adaptive immunity. Innate immunity acts as the body's first line of defense and does not discriminate between different types of pathogens. As a result, it has a general response that includes the use of physical barriers, such as the skin and mucous linings, as well as chemical and cell mediated responses (Turvey & Broide, 2010). Adaptive immunity or acquired immunity involve highly specialized cells and are specific to pathogens invading the body. It consists of two kinds of lymphocytes - T cells and B cells. Due to its general nature, the innate immune system is much quicker at inducing an inflammatory response than the adaptive immune system (Alberts B et al.,2002). However, both systems maintain mechanisms that are responsible for discerning between foreign and self antigens. Failure to do this lends itself to the adaptive system attacking the body's cells as though they were foreign and is generally the basis of autoimmune disorders (Marshall et al., 2018).

In the adaptive system, both B and T cells are derived from hematopoietic stem cells (HSC) in the bone marrow. T cells go on to develop in the thymus and are known as naive T cells while B cells remain in the bone marrow, mature there and aid with the production of antibodies (Bonilla & Oettgen, 2010). Naive T cells are important because they can eventually differentiate into different kinds of T cells including T helper cells (Th), T regulatory cells (Treg) and cytotoxic T cells. The adaptive system is also integral to the question of immunological memory by improving the efficiency with which the immune system responds to recurring pathogens. During an encounter with a pathogen, naive T cells are activated and become effector cells which release cytokines and recruit secondary effector T cells. After the pathogen of interest is eliminated, most of these effector T cells die off in what is known as contraction (Gasper et al., 2014). However, some persist holding the memory of the pathogen and are able to expand remarkably quicker than the initial 1 to 2 day priming period that naive memory cells require. (Jelley-Gibbs et al., 2000). The understanding of memory cells has become vital to vaccination development making them an increasingly relevant area of interest (Kaech et al., 2002). This is especially true with the COVID-19 pandemic as the knowledge of memory cells has been used to develop and track the efficacy of COVID-19 vaccines (Woldemeskel et al., 2021).

As briefly mentioned earlier, T cells begin their development as HSCs before traveling to the thymus, a gland located behind the sternum and in between the lungs, for development. It is in the thymus that these cells will interact with MHC-I or MHC - II complexes displayed on professional antigen presenting cells (APCs), allowing them to eventually express surface molecules CD4 or CD8 (Luckheeram et al., 2012). APCs are cells that communicate information and mediate cell responses between the innate and adaptive system through the presentation of antigens (Gaudino & Kumar, 2019). Cells that express CD4 and interact with MHC -II complexes help with cytokine production and are known as T helper or effector cells (Luckheeram et al., 2012). T cells that express CD8 and interact with MHC - I are known as cytotoxic because they proliferate and induce cell death of targeted and infected cells (Bevan, 2004).

At this point the T cells are still characterized as naive. In the case of CD4 cells, they interact with the MHC-II complex alongside TCR to begin T cell differentiation and induce the proliferation of more naive T cells. Some T helper cell differentiations include Th1, Th2 and Th17 and they are often separated according to the type of cytokine they produce (Luckheeram et al., 2012). Th1 produces mainly interleukin 12 (IL-12) and interferon γ (IFγ) which works to increase proinflammatory response to pathogens. Th 2 mainly produces interleukin 4,5,10 and 13 and generally works to promote an antiinflammatory response to balance out Th1 cytokine production. It is also implicated in atopy which has to do with the body's allergic response (Berger, 2000). Th 17 produces cytokine IL-17A and IL-17F which also have proinflammatory and autoimmune properties (Gagliani et al., 2015).

In the case of these effector Th1 cells, when activated they can respond to pathogens or foreign bodies after which many of them die off. However, a small number of them show immunological resilience and become memory T cells, being able to respond to similar pathogens much faster if they were to show up again. They are also able to travel more efficiently through tissue and proliferate faster (Pennock et al., 2013).

Although T cells are initially produced in the bone marrow and achieve maturation in the thymus, they migrate to secondary peripheral lymphoid organs to exert their function. This is particularly critical at. mucosal tissues, such as the intestine, which are in direct contact with the environment (Ma et al., 2019). The intestine is a critical site of immune tolerance and protection - relevance to generation of tolerance to food and benign microbes, and protection from pathogens (Zheng et al., 2020). Naive T cells migrate from the thymus to gut associated lymphoid tissue (GALT) where they interact with antigen presenting cells (APCs). This interaction results in the activation of T cells and leads to the upregulation of gut homing molecules such as the integrin a4b7, chemokine receptor CCR9, activation markers and adhesion

molecules - some of which I will expand on later. The combination of these molecules also dictates where these cells will migrate to in the intestine. (Ma et al., 2019)

T cell production begins *in utero* and most abundantly proliferates in early childhood. (Saule et al., 2006; Semmes et al., 2021) However, most of these neonatal T cells will be naive and many have also been found to be regulatory T cells (Treg). These are T cells that suppress immune responses and allow for self tolerance (Kondělková et al., 2010). They are especially vital in fetal immune systems by allowing for fetal maternal immune homeostasis and reducing the possibility of maternal immunological rejection (Huang et al., 2020). Treg cells can be found in parts of the fetal gut and periphery as early as the second trimester (Cupedo et al., 2005). This example of fetal naive T cells preferentially generating Treg cells illustrates the unique needs of the feral immune system as it encounters the outside world and a myriad of microbial exposures (Mold et al., 2008).

CD4+ PLZF+ Cells

Another primary population in the fetal immune system, specifically the small intestine, are CD4+ PLZF+ T cells. Significantly, they have been found to be a primary population in the cord blood of infants who have dealt with exposure to *in utero* inflammatory insults, suggesting these T cells contribute to fetal immune inflammation which is detrimental to the fetus and to the pregnancy.

Promyelocytic leukemia zinc finger protein (PLZF) is a transcription factor that has been found to affect immunological responses. It has been previously found to be necessary for the differentiation of invariant killer T cells (iNKT) (Kovalovsky et al., 2008). CD4+PLZF+ T cells make up the majority of memory T cells found in the human fetal intestine and have rapid effector function/rapid production of pro-inflammatory cytokines (Halkias et al., 2019; Weinreich et al., 2010). CD4+ PLZF+ memory T cells were found to secrete more Th1 cytokines such as IFNγ and TNFα than the usual CD4+ memory T cells which indicates a higher effector potential and pro-inflammatory response (Locher et al., 2022). This adds to the complex nature of the fetal immune system that is trying to balance the need for a proinflammatory

response against the predominant need for an immune system especially to the mother. Interestingly, this population of CD4+ PLZF+ memory T cells is specific to the fetal immune systems as they are essentially absent from the healthy adult intestine (Halkias et al., 2019) which makes it an interesting avenue to explore immunology especially in relation to the generation of early life immune protection. Additionally, more research into the upregulation of this population of memory T cells is needed to provide clinical treatment into fetal immune systems that might be lacking this vital group of T cells.

The Role of IL-7 and its Clinical Relevance

In addition to TCR signaling, cytokines are critical mediators of immunological responses. One important cytokine for T cells is IL-7 which is necessary at many different stages of T cell development, for example, the progenitor to naive T cell transition early on in the thymus. IL7 is produced by a range of cells including fetal liver cells, in the bone marrow, in the thymus and by epithelial cells - including intestinal epithelial cells (IELs) (Alpdogan & Vandenbrink, 2005). It binds to the IL-7 receptor, expressed on naive T cells, which allows it to activate important signaling through JAK proteins and the signaling molecule, STAT-5. This signaling is responsible for a cascade of effects including the regulation of anti-apoptotic genes such as Bcl-2 and Mcl-1 during the T cell selection process. IL-7 receptor expression is found in naive T cells as well as memory T cells (Mazzucchelli & Durum, 2007). Lack of IL-7 or IL-7 receptors can lead to severe immunodeficiency and complete loss of T cells leading to increasing interest in potential cytokinetic-based therapy with IL-7 to reconstitute and encourage the expansion of T cell populations in lymphopenic patients. (Puel et al., 1998)

Markers of Interest:

a4b7

T cell migration to the GALT depends on interactions with integrins and chemokines. It is in the GALT that mucosal dendritic cells release retinoic acid which in turn upregulates integrins like a4b7 and

chemokine receptor CCR9 (van Wijk & Cheroutre, 2010). A4b7 is an integrin expressed on the T cell surface that interacts with the mucosal addressin cell adhesion molecule 1 protein (MADCAM -1) which is expressed primarily by intestinal epithelial cells (IECs)(Ma et al., 2019). This interaction allows for T cells that have migrated to the intestinal mucosa to attach to the tissue there and makes a4b7 essential for homing of T cells in the gut. This is further confirmed by the fact that a deficiency in a4b7 leads to break down of the GALT formation (Wagner et al., 1996). Previous research has demonstrated that cytokines, specifically IL-7, drives the upregulation of a4b7 in T cells in lymphopenic mice. (Cimbro et al., 2012)

CXCR4

Another important aspect of gut homing are chemokine receptors and ligands. One such receptor is CXCR4 which is the chemokine receptor for the ligand CXCL12 or stromal cell derived factor - 1(SDF-1) (Thelen, 2001). It is one of a family of chemokine receptors implicated in T cell migration and development (Contento et al, 2008). It is also broadly expressed by more subsets of T cells than some other chemokine receptors such as CCR9 (Marelli-Berg et al, 2008). CXC12 is expressed primarily in IECs which suggests that it is important for migration from the thymus (Heidemann et al., 2004). However, there has been conflict over the precise role of CXCR4 in the migration of T cells in response to inflammation (Abbal et al., 1999; Carroll et al., 1997). Despite this conflict over the role of CXCR4, blocking of the CXCR4 and CXCL12 systems has led to significant loss of T cell adhesion in the gut. (Oyama et al., 2007) Additionally, CXCR4 and CXC12 have also been linked to upregulation a4b7 which further confirms its role in gut homing (Wright et al., 2002). Furthermore, IL-7 has been identified as a cytokine that drives CXCR4 upregulation which is important as CXCR4 usually downregulates after binding with its ligand as a form of self regulation. (Schmitt et al., 2003). The coexpression of a4b7 and CXCR4 substantiates its role in the gut homing process for naive T cells. It also means that IL-7 presents a tool, with which we can upregulate CXCR4 expression in lacking immune systems.

CD45RO and CD45RA

One way of identifying memory cells is through expression of the surface molecule tyrosine phosphatase CD45 splice variants which are differentially expressed on activated naive and effector T cells (Arlettaz et al., 1999). Lymphocytes express two isoforms in the human body which are indicators of different stages of T cell development - CD45RA and CD45RO. Naive T cells are defined by expression of CD45RA and memory T cells expressing CD45RO (Arlettaz et al., 1999). Additionally, memory T cells have been found to down regulate the CD45RA marker (Saule et al., 2006) while CD45RO has been found to be upregulated in memory (Miyawaki et al., 1990; Valentine et al., 2013). IL-7 has been found to induce the memory T cell phenotype in naive T cells after 48 hours of stimulation (Cimbro et al., 2012). After 12 days of stimulation in IL-7, there has been marked upregulation of CD45RO expression with or without T cell receptor signaling (TCR). However, generally more CD45RO expression is seen with higher concentrations of TCR (Locher et al., 2022).

The Experiments

In this experiment, I investigate the mechanisms involved in the gut homing of naive T cells through the expression of a4b7 and CXCR4. These two markers have been found to be integral to migration and homing of naive T cells in the gut and have been found to be upregulated in the presence of the cytokine IL-7. I also look at the transition from naive to memory cells by tracking expression of the CD45 isoforms - CD45RA and CD45RO. CD45RA is commonly seen on naive T cells while CD45RO has been commonly associated with memory T cells. To address these two questions, I construct a timeline of the markers CD45RO, CD45RA, CXCR4 and a4b7 over the course of 7 days in the presence of 4 cytokine conditions - IL-7, IL-7 with TCR, IL-2 and no cytokines. I determined whether CD4+PLZF+ naive T cells upregulated gut homing markers in the presence of IL-7 and if they acquired the CD45RO marker in the presence of IL-7 alone. Finally, I determined the rate at which this acquisition occurs, a question which has not yet been fully explored by prior research.

By understanding the timeline I hope to better understand the signals that instruct T cell maturation and migration once they leave the thymus. Examination of CD45RA and CD45RO expression will be coupled to the acquisition of other gut homing associated markers such as a4b7 which mediate naive T cell ability to adhere to endothelial cells in the intestine and CXCR4 which is important for helping newly emerging T cells out of the thymus and directing them to the periphery.

Methods

In these experiments, I modified the methods and procedures described in Locher et al (2022).

Cell Isolation

Fetal thymocyte samples were collected and processed within 2 hours of collection. The thymocytes were pressed through a 70μm strainer in order to obtain single cell suspensions. The cells were then cryopreserved in freezing medium (90% FBS + 10% DMSO) at -80C. For this experiment, 9 samples were selected and thawed. Since these cells had been cryopreserved for over a year, cells were manually counted using Trypan Blue (Sigma Aldrich) both before and after the biotin selection to enumerate viable cells. Our population of interest was single positive CD4+ T cells. In order to ensure that I only got single positive CD4 T cells and not CD4+ CD8+ thymocyte intermediate cells, a negative biotin selection was conducted to select against developmental markers. Single positive (SP) CD4+ were enriched to >90% purity by adapting the EasySep Biotin Positive Selection Kit from STEMCELL Technologies to instead perform a negative selection (and keeping the unbound cell fraction as our population of interest). The lineage and developmental markers I then selected against were CD56, CD16, CD14, CD11c, CD20, CD1a, CD34, and CD8 leaving only SP CD4 T cells as our cells of interest to be poured off and collected for further analysis.

Naïve CD4 T cell Cytokine Assay

SP CD4 thymocytes were isolated as the source of the naïve CD4 T cells. Since these cells are extracted from the thymus they are unequivocally a source of naïve T cells and therefore a good candidate population to study their differentiation into memory T cells. The cells are then reconstituted in 200ml of cells and cytokine solution, plated in 7 96-well plates with a density of 30K-80K CD4+ cells/well with duplicates for each condition. Cytokine solutions were diluted with complete RPMI (cRPMI) supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, and 1Xf nonessential amino acids in order to produce desired concentrations. Cells were then cultured with IL-7 (5 ng/mL), IL-7 and TCR stimulation which was achieved through IL-7 (2 ng/mL) and soluble αCD28 mAb (2 μg/mL) (CD28.2, Invitrogen, cat#MA1-20792) with plate-bound αCD3 mAb (1 μg/mL) (clone HIT3a, BioLegend, cat#300313), IL-2 (10 ng/mL) and only cRPMI. Cells were cultured for up to 7 days with media changes every 2-3 days. Cells were kept at 37°C in 4% O2 for the duration of the experiment and each day, one of the marked 96 well plates was taken out for immunostaining and both surface and intracellular in order to build a timeline of marker development. Cells were fixed and permeabilized before being stored at -20C. Flow cytometry was then performed every 2-3 days and marker expression was collected for each day of the 7 days.

Antibodies and Flow Cytometry

Cells were stained with a surface stain master mix that included human Fc block (STEMCELL Technologies, cat#18520) as well as mouse and rat anti-human monoclonal antibodies (mAbs) including Aqua LD (Invitrogen, cat# L34957), CD45RO BV650 (clone UCHL1, BioLegend, cat# 304232), CCR7 BV421 (clone G043H7, BioLegend, cat# 353208), **β**7 PE (clone FIB504, BioLegend, cat#321204), TCRγδ CF594 (clone B1, BD Horizon, cat#562511), CD45RA PeCY7 (clone HI100, BD Pharmingen, cat#560675), CD49d/α4 BV605 (clone 9F10, BioLegend, cat#304324), CXCR4/CD184 BUV395 (clone HI30, BD Horizon, cat#563792), CD3 BUV737(clone UCHT1, BD Horizon, cat#612750). The cells were then fixed and permeabilized using the BioLegend FOXP3 Fix/Perm Buffer Set. Finally, cells were intracellularly stained with mouse and rat anti-human monoclonal antibodies including TCRαβ Perp-e710

(clone IP26, Invitrogen, cat# 46-9986-42), CD45RA PE-Cy7 (clone HI100, BD Biosciences, cat# 560675), CD4 APC-H7 (clone L200, BD Biosciences, cat# 563865), PLZF APC (clone 6318100, R&D, cat# IC2944A), CD161 BV711 (clone DX12, BD Biosciences, cat# 563865), CD45 BUV395 (clone HI30, BD Biosciences, cat# 563792), BCl2 FITC (clone 100, BioLegend, cat#658704),

Cells were then taken for flow cytometry. All data was acquired with an LSR/Fortessa Dual SORP flow cytometer (BD Biosciences) and analyzed with FlowJo V10.0.8 (TreeStar) software.

Data Processing

Data was processed using FlowJo to obtain our population of interest. Cells were gated in order to look at the CD4 PLZF+ and CD4 PLZF- population. Marker development was then plotted using ggplot in R studio. We generated boxplot and flow graphs of the a4b7, CD45RO, CXCR4 production over a 7-day period. Box plot upper and lower hinges represent the first and third quartiles, the center line indicates the median, and the whiskers extend from the hinge to the highest and lowest value no further than 1.5 (interquartile range) from the hinge.

Results

Overview:

To address our questions, I divided the results into 3 sections. In Section 1, I looked at whether or not IL-7 was successful in upregulating a4b7 and CXCR4, which previous literature suggests, and at what rate this upregulation occurs. In Section 2, I looked at the transition from naive to memory T cells as tracked by the expression of the CD45RO marker. Below I will discuss the data patterns and their interpretations. Unfortunately I do not have access to the data to conduct the statistics necessary to confirm the significance of the data patterns. Thus, my discussion of the results will only be qualitative.

1.1 IL-7 Drives Upregulation of the Integrin a4b7 in Prenatal PLZF+ CD4+ T cells

Previous literature has confirmed that IL-7 induces increased expression of the integrin a4b7 in naive T cells which is an integral integrin for gut homing and T cell differentiation (Cimbro et al, 2012). However, the effects of IL-7 on a4b7 production in our population of PLZF+CD4+ T cells versus PLZF- CD4+ T cells have yet to be fully understood. To determine whether or not PLZF+CD4+ T cells showed a variation in a4b7 production and to determine the rate at which this expression occurs, I tracked a4b7 production for 7 days, using flow cytometry to plot a4b7 expression on each day.

In Fig 1A, we present all the data from all three runs collated together. I plot a4b7 frequencies by day, cytokine condition and type of run. Three runs of this experiment were conducted. Each row depicts all the results for one run. There were 4 cytokine conditions categorized horizontally - IL7, IL7 with TCR, IL2 and no cytokines. The x-axis shows the number of days that have passed by in each condition and the y- axis depicts the percentage of CD4+ PLZF+ cells that are expressing a4b7.

In Fig 1B, I show the a4b7 expression on Day 0 and Day 3. The cytokine conditions are displayed horizontally:IL-7, IL-7 with TCR, IL-2 and no cytokines. Each row represents one run. The y- axis depicts the time and y- axis depicts the percentage of CD4+ PLZF+ cells that are expressing a4b7 on the indicated days.

In Fig 1C, all runs were combined to show the data for the overall experiment by condition in order to illustrate the main data trends. The x- axis compares Day 1 versus Day 3 and the y-axis depicts the percentage of CD4+ PLZF+ cells that are expressing a4b7. In Fig 1D, a4b7 expression in the PLZF+ population is plotted against the PLZF- population after 3 days of stimulation in IL7. The x- axis indicates whether or not the population is PLZF + or - and the y axis depicts the percentage of CD4+ PLZF+ cells that are expressing a4b7.

Examination of the data in Fig 1A suggests that there is an increase in a4b7 in response to IL-7 post stimulation (Day 7 or d7 on Fig 1) than in comparison to pre-stimulation (d0). I demonstrated that the majority of this a4b7 expression occurs within 3 days of IL-7 stimulation. (Fig 1A), confirming that IL-7

drives rapid expansion of a4b7. To better understand the trend of upregulation of a4b7, I looked at the peaks of a4b7 expression, which was determined to be d3. Here there appears to be upregulation in both IL-7 and IL7 with TCR (Fig 1B, C). There does not seem to be much of a difference with or without TCR (Fig 1D).

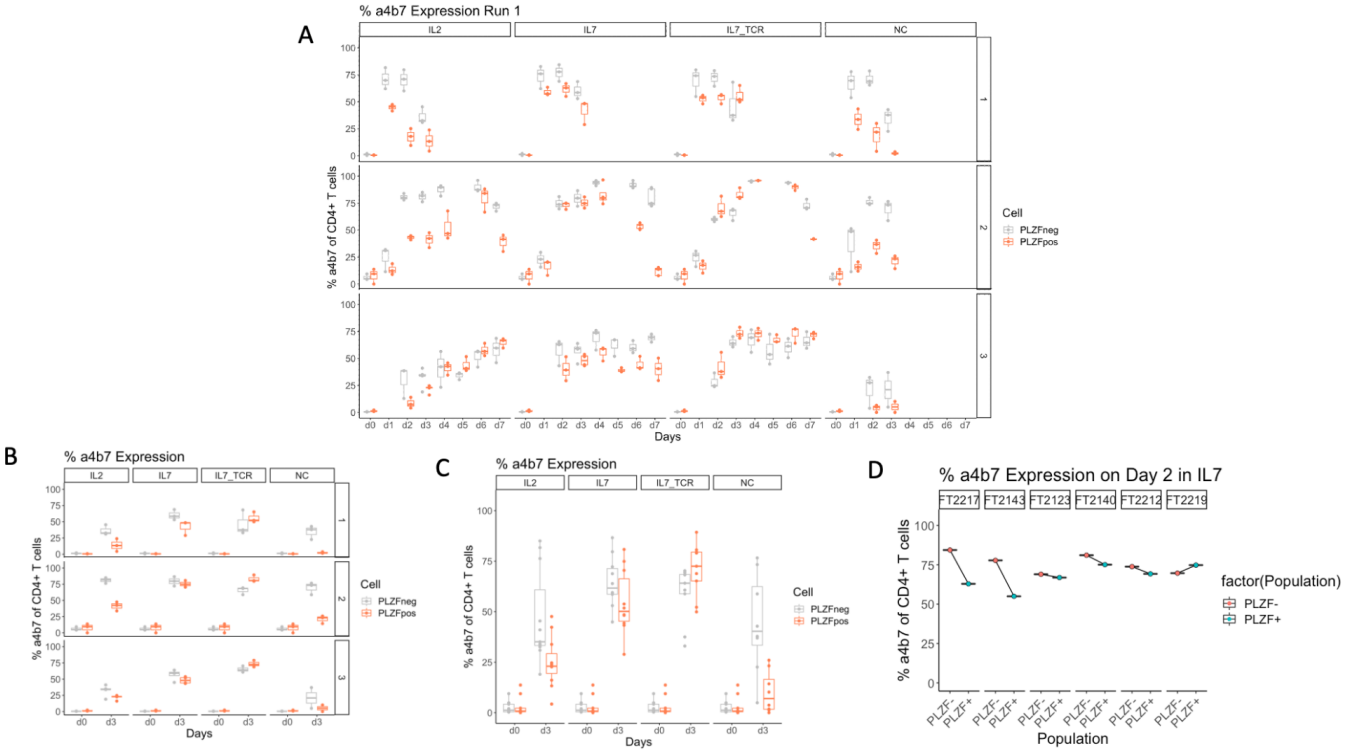

Fig 1:

(A) Representative flow plots of a4b7 expression by CD4+PLZF+ T cells after 7 days of cytokine stimulation. (B) A flow plot of a4b7 expression on only Day 1 and Day 3. (C) A collated flowplot of a4b7 expression on Day 1 and day 3 in the presence of different cytokine conditions.(D) a4b7 expression in the PLZF+ population is plotted against the PLZF- population after 2 days of stimulation in IL7.

The data from these graphs further illustrate that IL-7 with or without TCR is enough to increase upregulation of a4b7 in CD4+PLZF+ cells. Additionally, this upregulation has a quick onset, reaching its peak within 3 days of stimulation. This is apt to its role as a gut homing integrin for PLZF+CD4+ T cells as IL-7 drives the upregulation of a4b7 expression.

1.2 T Cell Migration: IL-7 Drives Upregulation of the Integrin CXCR4 in Prenatal PLZF+ CD4+ T cells

IL-7 drives CXCR4 production in T cells, but the speed at which this occurs has not been detailed closely (Schmitt et al, 2003). To determine this, I tracked CXCR4 expression for 7 days, using flow cytometry to plot CXCR4 expression on each day.

In Fig 1A, 3 runs were conducted, with each row depicting all the results for one run. There were 4 cytokine conditions categorized horizontally: IL-7, IL-7 with TCR, IL-2 and no cytokines. The x-axis shows the number of days that have passed by in each condition and the y-axis depicts the percentage of CD4+ PLZF+ cells that are expressing CXCR4 at the time of imaging. In Fig 1B, I show the CXC4 expression on Day 0 and Day 3. The cytokine conditions are displayed horizontally: IL-7, IL-7 with TCR, IL-2 and no cytokines. Each row represents one run. The x-axis indicates the time and y0axis depicts the percentage of CD4+ PLZF+ cells that are expressing CXCR4 on the indicated days. In Fig 1C, all these runs were combined to show the data patterns for the overall experiment by condition. The x-axis compares Day 0 against Day 3 and the y-axis depicts the percentage of CD4+ PLZF+ cells that are expressing CXCR4. In Fig 1D, I show a flow plot of CXCR4 expression in the PLZF+ population plotted against the PLZF- population after three days of stimulation in IL7. Here the x-axis displays the two times: Day 0 (start of the experiment) and Day 3 (day of peak production). The y-axis shows us the percentage of CD4+ PLZF+ cells that are expressing CXCR4 on each day. This graph is representative of all three runs.

There was an increase in CXCR4 expression in response to IL-7 post stimulation (d7) than in comparison to pre-stimulation (d0) (Figure 2A). I demonstrated that the majority of this CXCR4 expression occurs within 3 days of IL-7 stimulation (Fig 2A,) confirming that IL-7 drives the most rapid expression of CXCR4. To better understand the trend of upregulation of CXCR4, I looked at the peaks of CXCR4 expression, which was determined to be Day 3. Here I see upregulation in IL-7 alone specifically, more so than in IL7 with TCR or in IL-2 (Fig 2B, C).

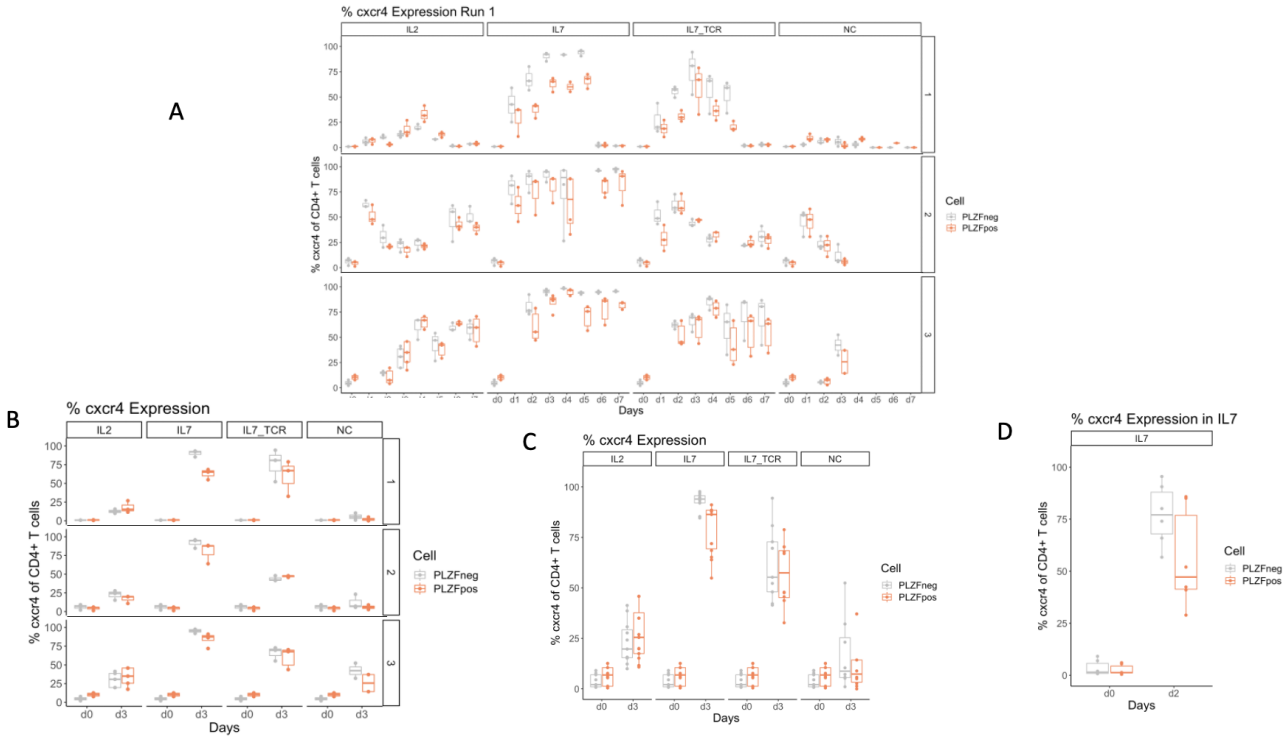

Fig 2:

(A) Representative flow plots of CXCR4 expression by CD4+PLZF+ T cells after 7 days of cytokine stimulation. (B) A flow plot of CXCR4 expression on d0 and d3 in the presence of different cytokine conditions separated by run. (C) A collated flowplot of CXCR4 expression on d0 and d3 in the presence of different cytokine conditions for the overall experiment. (D) A flow plot of CXCR4 expression in the PLZF+ population plotted against the PLZF- population after 3 days of stimulation in IL7.

These results suggest that IL-7 alone is the optimal cytokine condition to upregulate CXCR4 expression in CD4+PLZF+ cells. As CXCR4 is important for migration to the periphery and mucosal tissue, IL-7 presents a method through which CD4+PLZF+ T cells can be sent to specific parts of the intestine.

2.0 Naive to Memory: Prenatal PLZF+ CD4+ T Cell Reacquire of the Memory Phenotype after IL-7 and IL-2 Stimulation

CD45RO+ has been shown to be both a developmental and memory marker that naive T cells will reacquire upon leaving the thymus and migrating to a periphery organ. Prior experiments have shown that IL-7 alongside CD3/CD28 and Il-7 alone is sufficient to stimulate the acquisition of CD45RO+, the memory phenotype (Locher et al, 2022).

In Fig 3, I show line graphs of CD45RO expression with each row representing a different condition: IL-7, IL-7 with TCR and IL2. Each number above represents a different thymic sample and the x-axis shows the timeline runs from d0 through d7. The y-axis depicts the percentage of CD4+ PLZF+ cells that are expressing CD45RO, which is the memory cell marker. There are 9 samples overall leading to 9 different timelines in the presence of each cytokine condition. Within each sample, I see all 7 days of marker expression. PLZF+ versus PLZF- are also plotted on this graph as well with PLZF+ in orange and PLZF- in gray to provide visual baseline controls.

Here I charted the timeline of this reacquisition to determine how quickly the acquisition of CD45RO+ marker occurred, therefore aiding us in our understanding naive to memory T cell activation.

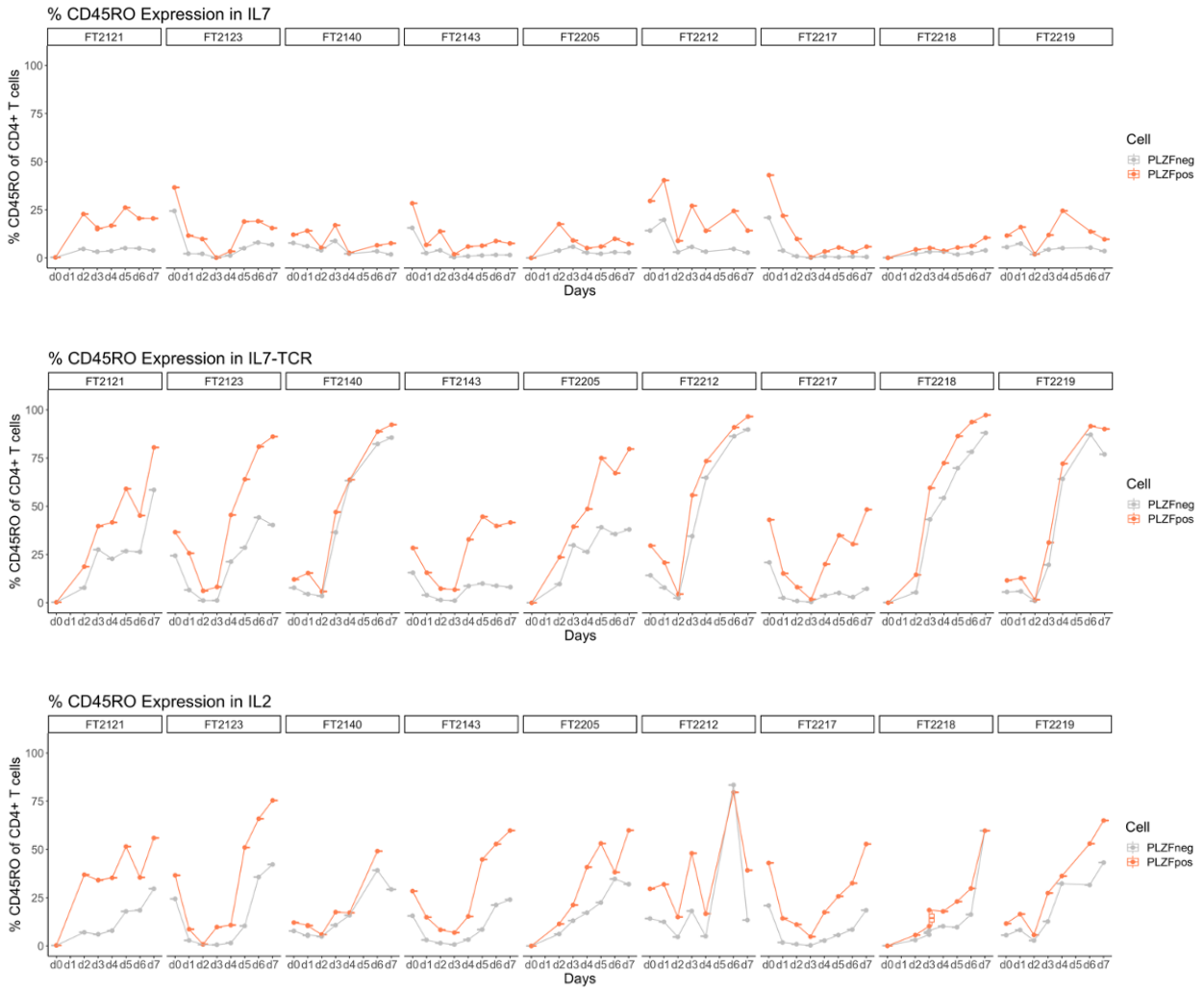

Fig 3:

CD45RO expression. Each row represents a different condition: IL-7, IL-7 with TCR and IL2. Although I expected IL-7 to show significantly increased acquisition of CD45RO+, I found highly variable data. This variability suggests that while the acquisition occurs, it is much less in the presence of IL-7 versus in the presence of IL-7 with TCR signaling and in IL- 2. This presents an avenue of interest whereas previous research has only seen major CD4+ PLZF+ expansion with stimulation with IL-7 (Locher, 2022) While IL-2 provides less expansion of CD4+ PLZF+ memory T cells, it still seems to offer increased expression of the memory phenotype.

Discussion

In this experiment I tracked the expression of a4b7, CXCR4 and CD45RO in CD4+ PLZF+ cells for 7 days in the presence of IL-7, IL-7 with TCR, IL-2 and no cytokines. Our results suggest that IL-7 and IL-7 with TCR are successful in driving upregulation of a4b7, with the most change occurring within 3 days of initial stimulation. Here, the most upregulation was observed in the presence of IL-7 with TCR. Additionally, IL-7 and IL-7 with TCR drive upregulation of CXCR4 with the most increased expression happening 3 days after stimulation. The most upregulation occurred in the presence of IL-7 alone. This shows that IL-7 with or without TCR signaling is integral to the increased expression of gut homing mechanisms from initial migration to adhesion in the intestinal mucosa. Furthermore, these changes occur very quickly after initial stimulation, making IL-7 an efficient tool in upregulating and strengthening the gut homing mechanism for T cells and repopulating a potentially depleted immune system.

I also found that IL-7 with TCR successfully drives the transition of naive cells to memory through a rapid increase in CD4+ PLZF+ T cells expressing the memory phenotype, the CD45RO+ marker. This confirms the research done by Locher, 2022. The results for IL-7 alone were far more variable and although some increases Ire observed, they Ire much less than with IL7 and TCR. It is possible that a longer period is needed to see more increase in CD45RO+ and a future experiment might consider extending the length to 14 days.

Although IL-2 was initially chosen as a negative control as it had not previously in literature shown significant results in expanding CD4+ PLZF+ cells (Locher et al., 2022), it did yield some notable increase in CD45RO+ expression, making it a potential candidate for aiding in the transition from naive T cells to memory. However, while in comparison to IL-7, IL-2 did not induce major expansion of CD4+ PLZF+ T cells, they still produce more IL-2 than PLZF- T cells (Halkias et al., 2019). Future research might help to clarify the relationship between IL-2 and the expression of the memory phenotype.

This provides an avenue for future clinical treatment with IL-7 and IL-2, especially in fetuses that are showing signs of immunodeficiency. By treating them with IL-7, there is a possibility that it can aid in the homing of their T cells which can allow for the later transition into memory T cells. A mechanism which is vital for early defense against pathogens and formation of the immune system. This is time sensitive since most thymic function occurs in early life, so ensuring the early introduction of these cytokines in a lacking immune system is critical (Kumar et al., 2019).

The use of cytokines alone to induce the memory phenotype in T cells through TCR signaling and the expression of CD45RO is a relatively quite novel idea. “True” memory T cells are formed after initial interaction with an antigen, allowing for the body to remember and react much more quickly to a pathogen it has already encountered. While cytokines have always been a part of the memory activation process, the lack of a specific antigen is a key difference. In the case of cytokine induced memory phenotypes - no such antigen has been encountered. However, using cytokine driven techniques can induce more T cells with the memory phenotype than would ordinarily be possible - meaning more production of target Th cytokines. Additionally, in some cases the memory phenotype can be induced without additional TCR signaling, reducing interaction with TCRs as a limiting factor (Geginat et al., 2001).

Indeed clinical administration of IL-7 to HIV -1 patients has already yielded replication of these results with a marked a4b7 and CXCR4 upregulation in naive T cells in vivo (Cimbro et al., 2012). Additionally, there was overall increased T cell recruitment of CD4+ and CD8+ T cells (Sereti et al., 2009). This is especially important as the gut has been identified as a primary location of CD4+ depletion during all stages of HIV infection (Church, 2005). Memory cells in the GALT have also been identified with having lower a4b7 expression in people with HIV-1, suggesting that this may be one of the mechanisms through which such depletion occurs. (Wittner et al., 2019) While the age group for this trial was mainly between 39 and 48, cytokine therapy provides an avenue of treatment that might be especially transformative for

younger children and fetuses living with HIV of which as of 2022, UNAIDS estimates there are 2.7 million worldwide. (UNAIDS., 2022)

Other molecules that might prove integral to track include retinoic acid which induces upregulation of a4b7 and consequently CXCR4. Additionally, more detailed timelines of the Th1 cytokines IFNγ and TNFα that CD4+ PLZF+ produce more of might also be helpful to track. Future research should continue to work on not just determining upregulation or downregulation but also the speed with which these molecules can be induced. This furthest opens up the possibility of clinical application and perhaps a deeper look into efficacy and even dose dependence.

Overall, the use of IL-7 to reconstitute fetal immune systems lacking a substantial CD4+ PLZF+ memory T cell population is a major finding that may help aid lymphopenic immune system and improve birth and early life outcomes. The efficiency and speed with which IL-7 stimulates the upregulation of vital gut homing markers, identifies it as a promising agent for cytokine therapy. The application of memory cells continues to be key in building up early life immunity and reducing the likelihood of sustained damage from repeat encounters with a pathogen. By understanding the role of IL-7 in fetal memory cell activation and maturation, we can further build our framework of the fetal immune system and aid members of our most vulnerable population during critical early life.

Acknowledgement:

I would like to thank everyone at the Halkias Lab - Dr. Joanna Halkias, Stephanie Makredes, Sara Park, and Margareta Mayer - for helping me to find my footing at UCSF, run this experiment and analyze the data. Your mentorship and support have been invaluable thus far. At CMC, I would like to thank Professor Catherine Reed for her meticulous attention to detail and many many sticky notes. I would also like to extend my gratitude to my advisor Dianna Graves, who has been incredibly supportive of my time at

CMC, Jess Neilson, and everyone at the Dean of Students Office who has made my attending Claremont McKenna College possible in the face of unpredictable challenges. To every friend, who's listened to talk for hours about IL-7 or listened patiently while I tried to parse out fetal immunology, thank you for your patience. Learning about fetal immunology has been a huge learning curve but majorly possible due to peer learning. Finally, I would like to thank my favorite humans on earth - my parents - for their constant love and kindness throughout my college experience. I could not have completed this without you.

References

Locher, V., Park, S., Bunis, D., Makredes, S., Burt, T. D., Fragiadakis, G. K., & Halkias, J. (2022). Homeostatic cytokines reciprocally modulate the emergence of prenatal effector PLZF+CD4+ T cells in humans. https://doi.org/10.1101/2022.09.19.508328

Halkias, J., Melichar, H.J., Taylor, K.T. *et al.* Tracking migration during human T cell development. *Cell. Mol. Life Sci.* 71, 3101–3117 (2014). https://doi.org/10.1007/s00018-014-1607-2

Halkias, J., Rackaityte, E., Hillman, S. L., Aran, D., Mendoza, V. F., Marshall, L. R., MacKenzie, T. C., & Burt, T. D. (2019). CD161 contributes to prenatal immune suppression of IFN-γ–producing PLZF+ T cells. *Journal of Clinical Investigation*, *129*(9), 3562–3577. https://doi.org/10.1172/jci125957

Cimbro, R., Vassena, L., Arthos, J., Cicala, C., Kehrl, J. H., Park, C., Sereti, I., Lederman, M. M., Fauci, A. S., & Lusso, P. (2012). IL-7 induces expression and activation of integrin α4β7 promoting naive T-cell homing to the intestinal mucosa. *Blood*, *120*(13), 2610–2619. https://doi.org/10.1182/blood-2012-06-434779

Contento, R. L., Molon, B., Boularan, C., Pozzan, T., Manes, S., Marullo, S., & Viola, A. (2008). CXCR4–CCR5: A couple modulating T cell functions. *Proceedings of the National Academy of Sciences*, *105*(29), 10101–10106. https://doi.org/10.1073/pnas.0804286105

Marelli-Berg, F. M., Cannella, L., Dazzi, F., & Mirenda, V. (2008). The Highway Code of T cell trafficking. *The Journal of Pathology*, *214*(2), 179–189. https://doi.org/10.1002/path.2269

Schmitt, N., Chêne, L., Boutolleau, D., Nugeyre, M.-T., Guillemard, E., Versmisse, P., Jacquemot, C., Barré-Sinoussi, F., & Israël, N. (2003). Positive regulation of CXCR4 expression and signaling by interleukin-7 in CD4 + mature thymocytes correlates with their capacity to favor human immunodeficiency X4 virus replication. *Journal of Virology*, *77*(10), 5784–5793. https://doi.org/10.1128/jvi.77.10.5784-5793.2003

Kumar, B. V., Connors, T. J., & Farber, D. L. (2018). Human T cell development, localization, and function throughout life. *Immunity*, *48*(2), 202–213. https://doi.org/10.1016/j.immuni.2018.01.007

Saule, P., Trauet, J., Dutriez, V., Lekeux, V., Dessaint, J.-P., & Labalette, M. (2006). Accumulation of memory T cells from childhood to old age: Central and effector memory cells in CD4+ versus

effector memory and terminally differentiated memory cells in CD8+ compartment. *Mechanisms of Ageing and Development*, *127*(3), 274–281. https://doi.org/10.1016/j.mad.2005.11.001

Yang, Q., Jeremiah Bell, J., & Bhandoola, A. (2010). T-cell lineage determination. *Immunological Reviews*, *238*(1), 12–22. https://doi.org/10.1111/j.1600-065x.2010.00956.x

Kaech, S. M., Wherry, E. J., & Ahmed, R. (2002). Effector and memory T-cell differentiation: Implications for vaccine development. *Nature Reviews Immunology*, *2*(4), 251–262. https://doi.org/10.1038/nri778

Pennock, N. D., White, J. T., Cross, E. W., Cheney, E. E., Tamburini, B. A., & Kedl, R. M. (2013). T cell responses: Naïve to memory and everything in between. *Advances in Physiology Education*, *37*(4), 273–283. https://doi.org/10.1152/advan.00066.2013

Ma, H., Tao, W., & Zhu, S. (2019). T lymphocytes in the intestinal mucosa: Defense and tolerance. *Cellular & Molecular Immunology*, *16*(3), 216–224. https://doi.org/10.1038/s41423-019-0208-2

van Wijk, F., & Cheroutre, H. (2010). Mucosal T cells in gut homeostasis and inflammation. *Expert Review of Clinical Immunology*, *6*(4), 559–566. https://doi.org/10.1586/eci.10.34

Semmes, E. C., Chen, J.-L., Goswami, R., Burt, T. D., Permar, S. R., & Fouda, G. G. (2021). Understanding early-life adaptive immunity to guide interventions for pediatric health. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.595297

Kovalovsky, D., Uche, O. U., Eladad, S., Hobbs, R. M., Yi, W., Alonzo, E., Chua, K., Eidson, M., Kim, H.-J., Im, J. S., Pandolfi, P. P., & Sant'Angelo, D. B. (2008). The BTB–zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature Immunology*, *9*(9), 1055–1064. https://doi.org/10.1038/ni.1641

Halkias, J., Rackaityte, E., Hillman, S. L., Aran, D., Mendoza, V. F., Marshall, L. R., MacKenzie, T. C., & Burt, T. D. (2019). CD161 contributes to prenatal immune suppression of IFN-γ–producing PLZF+ T cells. *Journal of Clinical Investigation*, *129*(9), 3562–3577. https://doi.org/10.1172/jci125957

Weinreich, M. A., Odumade, O. A., Jameson, S. C., & Hogquist, K. A. (2010). T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells. *Nature Immunology*, *11*(8), 709–716. https://doi.org/10.1038/ni.1898

Mazzucchelli, R., & Durum, S. K. (2007). Interleukin-7 receptor expression: Intelligent design. *Nature Reviews Immunology*, *7*(2), 144–154. https://doi.org/10.1038/nri2023

ALPDOGAN, O., & VANDENBRINK, M. (2005). IL-7 and IL-15: Therapeutic cytokines for immunodeficiency. *Trends in Immunology*, *26*(1), 56–64. https://doi.org/10.1016/j.it.2004.11.002

Puel, A., Ziegler, S. F., Buckley, R. H., & Leonard., W. J. (1998). Defective IL7R expression in T-B+NK+ severe combined immunodeficiency. *Nature Genetics*, *20*(4), 394–397. https://doi.org/10.1038/3877

Wagner, N., Löhler, J., Kunkel, E. J., Ley, K., Leung, E., Krissansen, G., Rajewsky, K., & Müller, W. (1996). Critical role for β7 integrins in formation of the gut-associated lymphoid tissue. *Nature*, *382*(6589), 366–370. https://doi.org/10.1038/382366a0

Thelen, M. (2001). Dancing to the tune of chemokines. *Nature Immunology*, *2*(2), 129–134. https://doi.org/10.1038/84224

Abbal, C., Jourdan, P., Hori, T., Bousquet, J., Yssel, H., & Pène, J. (1999). TCR-mediated activation of allergen-specific CD45RO+ memory T lymphocytes results in down-regulation of cell-surface

CXCR4 expression and a strongly reduced capacity to migrate in response to stromal cell-derived factor-1. *International Immunology*, *11*(9), 1451–1462. https://doi.org/10.1093/intimm/11.9.1451

Carroll, R. G., Riley, J. L., Levine, B. L., Feng, Y., Kaushal, S., Ritchey, D. W., Bernstein, W., Weislow, O. S., Brown, C. R., Berger, E. A., June, C. H., & St. Louis, D. C. (1997). Differential regulation of HIV-1 fusion cofactor expression by CD28 costimulation of CD4 + T cells. *Science*, *276*(5310), 273–276. https://doi.org/10.1126/science.276.5310.273

Oyama, T., Miura, S., Watanabe, C., Hokari, R., Fujiyama, Y., Komoto, S., Tsuzuki, Y., Hosoe, N., Nagata, H., & Hibi, T. (2007). CXCL12 and CCL20 play a significant role in mucosal T-lymphocyte adherence to intestinal microvessels in mice. *Microcirculation*, *14*(7), 753–766. https://doi.org/10.1080/10739680701409993

Wright, N., Hidalgo, A., Rodríguez-Frade, J. M., Soriano, S. F., Mellado, M., Parmo-Cabañas, M., Briskin, M. J., & Teixidó, J. (2002). The chemokine stromal cell-derived factor-1α modulates α4β7integrin-mediated lymphocyte adhesion to mucosal addressin cell adhesion molecule-1 and fibronectin. *The Journal of Immunology*, *168*(10), 5268–5277. https://doi.org/10.4049/jimmunol.168.10.5268

Heidemann, J., Ogawa, H., Rafiee, P., Lügering, N., Maaser, C., Domschke, W., Binion, D. G., & Dwinell, M. B. (2004). Mucosal angiogenesis regulation by CXCR4 and its ligand CXCL12 expressed by human intestinal microvascular endothelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *286*(6). https://doi.org/10.1152/ajpgi.00417.2003

Schmitt, N., Chêne, L., Boutolleau, D., Nugeyre, M.-T., Guillemard, E., Versmisse, P., Jacquemot, C., Barré-Sinoussi, F., & Israël, N. (2003). Positive regulation of CXCR4 expression and signaling by interleukin-7 in CD4 + mature thymocytes correlates with their capacity to favor human

immunodeficiency X4 virus replication. *Journal of Virology*, *77*(10), 5784–5793. https://doi.org/10.1128/jvi.77.10.5784-5793.2003

Cimbro, R., Vassena, L., Arthos, J., Cicala, C., Kehrl, J. H., Park, C., Sereti, I., Lederman, M. M., Fauci, A. S., & Lusso, P. (2012). IL-7 induces expression and activation of integrin α4β7 promoting naive T-cell homing to the intestinal mucosa. *Blood*, *120*(13), 2610–2619. https://doi.org/10.1182/blood-2012-06-434779

Arlettaz, L., Barbey, C., Dumont-Girard, F., Helg, C., Chapuis, B., Roux, E., & Roosnek, E. (1999). CD45 isoform phenotypes of human T cells: CD4+CD45RA–ro+ memory T cells re-acquire CD45RA without losing CD45RO. *European Journal of Immunology*, *29*(12), 3987–3994. https://doi.org/10.1002/(sici)1521-4141(199912)29:12<3987::aid-immu3987>3.0.co;2-4

Jelley-Gibbs, D. M., Lepak, N. M., Yen, M., & Swain, S. L. (2000). Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. *The Journal of Immunology*, *165*(9), 5017–5026. https://doi.org/10.4049/jimmunol.165.9.5017

Gasper, D. J., Tejera, M. M., & Suresh, M. (2014). CD4 T-cell memory generation and maintenance. *Critical Reviews in Immunology*, *34*(2), 121–146. https://doi.org/10.1615/critrevimmunol.2014010373

Saule, P., Trauet, J., Dutriez, V., Lekeux, V., Dessaint, J.-P., & Labalette, M. (2006). Accumulation of memory T cells from childhood to old age: Central and effector memory cells in CD4+ versus effector memory and terminally differentiated memory cells in CD8+ compartment. *Mechanisms of Ageing and Development*, *127*(3), 274–281. https://doi.org/10.1016/j.mad.2005.11.001

Valentine, M., Song, K., Maresh, G. A., Mack, H., Huaman, M. C., Polacino, P., Ho, O., Cristillo, A., Kyung Chung, H., Hu, S.-L., & Pincus, S. H. (2013). Expression of the memory marker CD45RO on helper T cells in macaques. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0073969

Miyawaki, T., Kasahara, Y., Taga, K., Yachie, A., & Taniguchi, N. (1990). Differential expression of CD45RO (UCHL1) and its functional relevance in two subpopulations of circulating TCR-gamma/delta+ lymphocytes. *Journal of Experimental Medicine*, *171*(5), 1833–1838. https://doi.org/10.1084/jem.171.5.1833

Sereti, I., Dunham, R. M., Spritzler, J., Aga, E., Proschan, M. A., Medvik, K., Battaglia, C. A., Landay, A. L., Pahwa, S., Fischl, M. A., Asmuth, D. M., Tenorio, A. R., Altman, J. D., Fox, L., Moir, S., Malaspina, A., Morre, M., Buffet, R., Silvestri, G., & Lederman, M. M. (2009). IL-7 administration drives T cell–cycle entry and expansion in HIV-1 infection. *Blood*, *113*(25), 6304–6314. https://doi.org/10.1182/blood-2008-10-186601

UNAIDS., J. O. I. N. T. U. N. I. T. E. D. N. A. T. I. O. N. S. P. R. O. G. R. A. M. M. E. O. N. H. I. V. A. N. D. A. I. D. S. (2022). *In danger: Unaids global aids update 2022*. UNITED NATIONS.

Church, J. A. (2005). CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *Pediatrics*, *116*(Supplement_2), 572–573. https://doi.org/10.1542/peds.2005-0698bbbb

Wittner, M., Schlicker, V., Libera, J., Bockmann, J.-H., Horvatits, T., Seiz, O., Kummer, S., Manthey, C. F., Hüfner, A., Kantowski, M., Rösch, T., Degen, O., Huber, S., Eberhard, J. M., & Schulze zur Wiesch, J. (2019). Comparison of the integrin α4β7 expression pattern of memory T cell subsets in HIV infection and ulcerative colitis. *PLOS ONE*, *14*(7). https://doi.org/10.1371/journal.pone.0220008

Woldemeskel, B. A., Garliss, C. C., & Blankson, J. N. (2021). SARS-COV-2 mrna vaccines induce broad CD4+ T cell responses that recognize SARS-COV-2 variants and HCoV-NL63. *Journal of Clinical Investigation*. https://doi.org/10.1172/jci149335

Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Innate Immunity.

Turvey, S. E., & Broide, D. H. (2010). Innate immunity. *Journal of Allergy and Clinical Immunology*, *125*(2). https://doi.org/10.1016/j.jaci.2009.07.016

Bonilla, F. A., & Oettgen, H. C. (2010). Adaptive immunity. *Journal of Allergy and Clinical Immunology*, *125*(2). https://doi.org/10.1016/j.jaci.2009.09.017

Marshall, J. S., Warrington, R., Watson, W., & Kim, H. L. (2018). An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology*, *14*(S2). https://doi.org/10.1186/s13223-018-0278-1

Gaudino, S. J., & Kumar, P. (2019). Cross-talk between antigen presenting cells and T cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.00360

Luckheeram, R. V., Zhou, R., Verma, A. D., & Xia, B. (2012). CD4+T cells: Differentiation and functions. *Clinical and Developmental Immunology*, *2012*, 1–12. https://doi.org/10.1155/2012/925135

Bevan, M. J. (2004). Helping the CD8+ T-cell response. *Nature Reviews Immunology*, *4*(8), 595–602. https://doi.org/10.1038/nri1413

Berger, A. (2000). Science commentary: Th1 and th2 responses: What are they? *BMJ*, *321*(7258), 424–424. https://doi.org/10.1136/bmj.321.7258.424

Gagliani, N., Huber, S., & Flavell, R. (2015). Intestinal regulatory CD4 + T cells. *Mucosal Immunology*, 777–785. https://doi.org/10.1016/b978-0-12-415847-4.00038-0

Kondělková, K., Vokurková, D., Krejsek, J., Borská, L., Fiala, Z., & Andrýs, C. (2010). Regulatory T cells (Treg) and their roles in immune system with respect to immunopathological disorders. *Acta Medica (Hradec Kralove, Czech Republic)*, *53*(2), 73–77. https://doi.org/10.14712/18059694.2016.63

Huang, N., Chi, H., & Qiao, J. (2020). Role of regulatory T cells in regulating fetal-maternal immune tolerance in healthy pregnancies and reproductive diseases. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.01023

Cupedo, T., Nagasawa, M., Weijer, K., Blom, B., & Spits, H. (2005). Development and activation of regulatory t?cells in the human fetus. *European Journal of Immunology*, *35*(2), 383–390. https://doi.org/10.1002/eji.200425763

Mold, J. E., Michaëlsson, J., Burt, T. D., Muench, M. O., Beckerman, K. P., Busch, M. P., Lee, T.-H., Nixon, D. F., & McCune, J. M. (2008). Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science*, *322*(5907), 1562–1565. https://doi.org/10.1126/science.1164511

Zheng, D., Liwinski, T., & Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Research*, *30*(6), 492–506. https://doi.org/10.1038/s41422-020-0332-7

Geginat, J., Sallusto, F., & Lanzavecchia, A. (2001). Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4+ T cells. *Journal of Experimental Medicine*, *194*(12), 1711–1720. https://doi.org/10.1084/jem.194.12.1711